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SIMON FLEXNER, M.D.

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# THE DETAILS OF THE PHOTOGRAPHICALLY RECORDED VENOUS PULSE IN AURICULAR FIBRILLATION.

BY WALTER L. NILES, M.D., AND CARL J. WIGGERS, M.D.

(From the Second Medical Division of Bellevue Hospital and the Physiological  
Laboratory of Cornell University Medical College, New York City.)

PLATES 1 TO 4.

(Received for publication, September 15, 1916.)

## INTRODUCTION.

The condition of auricular fibrillation is easily diagnosed by the aid of arterial and jugular tracings or by electrocardiograms, and in most cases it can be recognized by careful physical examination alone. It is not possible, however, to interpret all the waves found in the venous records. Generally, these tracings may be placed in one of two classes; (a) those in which prominent systolic waves, differing in contour from the small, impact waves of the normal phlebogram, predominate or occur alone, and (b) those in which large or small diastolic waves recur occasionally, in groups, or in a continued series averaging 250 to 500 per minute.

The prominent systolic waves, designated as ventricular waves, have generally been attributed to an associated tricuspid regurgitation. Lewis<sup>1</sup> states: "The ventricular form of venous pulse' is a term which expresses the only fixed quality manifested by graphic records taken from the jugular veins in these cases. . . . The older conception, that the prominence of the venous pulsation is an index of the degree of tricuspid reflux, is not without a definite foundation." The inference follows that tricuspid regurgitation is a frequent accompaniment of this form of arrhythmia. It is of interest to reexamine to what extent this is true.

The diastolic waves are often so recurrent and numerous that it is impossible to account for them under the assumption that the auricle is in a dilated and finely fibrillating state, the condition to which the term auricular fibrillation was first applied. Many electrocardiograms also show definite variations resembling P waves or diastolic wave groups which are interspersed between easily recog-

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<sup>1</sup> Lewis, T., Mechanism of the Heart Beat, London, 1911, 198.

nized ventricular complexes. Consequently it has been suggested (Hewlett and Wilson<sup>2</sup>) that in some cases a condition of coarse fibrillation obtains, or that the condition is associated with a state of rapid coordinated contractions designated as auricular tachyrythmia, or flutter (Jolly and Ritchie<sup>3</sup>). Concerning the diagnostic differentiation between flutter and fibrillation, Ritchie states:

"If polygraph tracings reveal a rhythmic series of large auricular waves in the jugular veins at times when the ventricles are in diastole, the condition is clearly flutter and not fibrillation. On the other hand, if the waves are small, irregular in rhythm, and very rapid, and if at the same time there is little uniformity in the grouping of arterial pulse beats, the auricles are either in fibrillation or in the form of activity representing simultaneous flutter and fibrillation."

Hewlett and Wilson,<sup>2</sup> in 1915, reviewed the experimental relation between fine and coarse fibrillation and comment upon the rarity with which large recurrent diastolic waves are found in venous tracings. They conclude that as a rule clinical fibrillation is of the fine type, but present a case, fully studied by photographic registration, in which distinct diastolic waves, which have no relation to electrocardiogram wavelets, were present. This they interpreted as a condition of coarse fibrillation.

A more comprehensive attempt to establish the extent to which coarse and fine fibrillation occur clinically and to what degree these in turn are associated with flutter is of apparent interest. For these reasons an objective analysis of the jugular and other records taken by photographically recording capsules, was undertaken, and their interpretation harmonized with the case histories and other physical signs present at the time.

### *Method of Procedure.*

Records of the venous pulse were taken simultaneously with the subclavian and radial pulses and with apex beats or electrocardiograms.<sup>4</sup> The superiority of capsules photographically recording the dynamic changes by reflecting beams of light need not be reviewed. The photographic records were obtained by mounting the Frank segment capsules, illuminating lamp, and photokymograph on a

<sup>2</sup> Hewlett, A. W., and Wilson, F. N., *Arch. Int. Med.*, 1915, xv, 786.

<sup>3</sup> Jolly and Ritchie, *Auricular Flutter*, New York, 1914, 125.

<sup>4</sup> We are indebted to Dr. Alexander Lambert for placing the electrocardiograph in Bellevue Hospital at our service and to Dr. Josephine B. Neal for valuable technical assistance in securing the clinical electrocardiograms.



table which could be wheeled to the bedside (Wiggers<sup>5</sup>). When simultaneous electrocardiograms were taken the table was also aligned with the projection system of a Cambridge model of Einthoven's string galvanometer. The movements of the segment capsule mirrors were thus photographed simultaneously with the shadow of the galvanometer string. Tracings of the jugular and radial pulses were also taken by a Jacquet polygraph for comparison with the photographic records.

### *Details of the Normal Photographic Curves.*

Although the details of the photographic venous pulse have often been described (Edens and Wartensleben,<sup>6</sup> Ohm,<sup>7</sup> Van Zwaluwenburg and Agnew,<sup>8</sup> and Wiggers<sup>9</sup>), our analysis of the photographic tracings in auricular fibrillation will be facilitated by a brief reconsideration of its chief features, especially as related to the photographic apex curves.

The two tracings shown in Fig. 1 give the essential characteristics of normal photographic records from the apex region and supraclavicular fossa. Experience has shown that there is nothing distinctive about the contour of the apex curve, which may in fact be positive or negative according to the position of the patient and placement of the tambour (Wiggers<sup>10</sup>). A detailed description is therefore superfluous. There is, however, one feature of these tracings which makes them valuable, for unless the chest wall is too thick or emphysematous lung overlies the heart, they show clear indications of the heart sounds (S<sup>1</sup> and S<sup>2</sup>) which enables a positive determination of the beginning and end of ventricular systole. The photographic venous pulse always shows three essential waves; to these a fourth wave is

<sup>5</sup> Wiggers, C. J., *J. Am. Med. Assn.*, 1915, lxiv, 1305.

<sup>6</sup> Edens, E., and Wartensleben, B., *Deutsch. Arch. klin. Med.*, 1911, civ, 552.

<sup>7</sup> Ohm, R., *Venenpuls- und Herzschallregistrierung als Grundlage für die Beurteilung der Mechanischen Arbeitsleistung des Herzens nach eigenen Methoden*, Berlin, 1914.

<sup>8</sup> Van Zwaluwenburg, J. G., and Agnew, J. H., *Heart*, 1911-12, iii, 343.

<sup>9</sup> Wiggers, *Modern Aspects of the Circulation in Health and Disease*, Philadelphia, 1915, 134; *J. Am. Med. Assn.*, 1915, lxiv, 1485.

<sup>10</sup> Wiggers, *Modern Aspects of the Circulation in Health and Disease*, Philadelphia, 1915, 151.

sometimes added when the cardiac cycle is long. The waves differ from polygraph curves in that each wave has a distinctive contour and that the relative prominence of the waves is different.

Since the records taken from cases of auricular fibrillation have so little in common with normal curves, it was found desirable to discontinue the commonly accepted terminology (a, c, v waves) introduced by Mackenzie. All records may readily be divided into systolic and diastolic portions. This is usually accomplished by the use of the two sound vibrations previously referred to in the apex tracing. Occasionally these vibrations are transmitted to the neck and superimposed on the venous tracings as well (Fig. 1, S<sup>2</sup>). The waves are best designated as presystolic, systolic, and diastolic. In the normal cycle each portion has, as a rule, a single wave. The presystolic wave (P) is associated with auricular systole. The systolic wave (S), occurring a considerable interval after the first sound, is synchronous with the subclavian rise of pressure and is, therefore, due to an arterial impact. The third wave (D<sub>1</sub>), commonly called v wave, is entirely diastolic in time, and is probably due to the cessation of stasis, as the tricuspid valve opens at its summit. When the cardiac cycle is long, a second diastolic wave (D<sub>2</sub>) is often found. Its significance cannot be positively given at present, but its presence in long cycles should be kept in mind in connection with fibrillation waves.

The following are average durations found for these waves:

	<i>sec.</i>
Presystolic wave (P), or a wave.....	0.09-0.10
Systolic wave (S), or c wave.....	0.28-0.32
First diastolic wave (D <sub>1</sub> ), or v wave.....	0.15-0.24
Second diastolic wave (D <sub>2</sub> ), or h wave.....	0.21-0.22

### *Types of Waves in Auricular Fibrillation.*

*Method of Analysis.*—The photographic tracings taken from cases of auricular fibrillation were analyzed in the same manner as normal records. The beginning and end of each ventricular systole must first be established. In many instances the first and second sound vibrations of the jugular or apex tracing satisfactorily determine these places; in others, where irregular cardiac action occurs, only a single sound is recorded in the jugular or apex tracing. Since



there is nothing distinctive about heart sound vibrations, it is occasionally impossible in auricular fibrillation to determine whether the vibrations represent the end of a premature but ineffective ventricular systole, a third sound, or a short diastolic murmur. The addition of an electrocardiograph record serves to elucidate these cases since the presence or absence of an R wave at these times differentiates them. Combined simultaneous records of the jugular pulse, apex beat, and electrocardiograms were therefore made in most cases, but as the electrocardiograms taken on bromide paper are not sufficiently clear to reproduce well, they have been omitted in the reproductions.

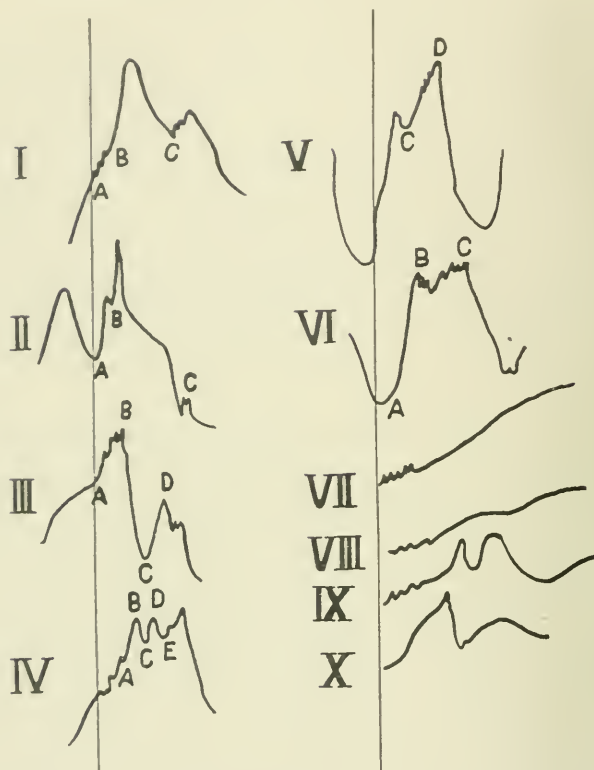
#### *Types of Waves Occurring during Ventricular Systole.*

In experimental auricular fibrillation many ventricular systoles occur that are too weak to open the semilunar valves, and hence fail to elevate aortic pressure, or elevate it so slightly that a pulse wave is not propagated to the more peripheral arteries (Wiggers<sup>11</sup>). The frequent occurrence of a pulse deficit is evidence that this also occurs in clinical fibrillation. The ventricular systoles may, therefore, be divided into effective and ineffective groups. Effective systoles may be recognized by the presence of a corresponding pulse wave in the subclavian or radial tracings. Ineffective systoles are often indicated by a single sound in the apex curve, but more certainly by the presence of a ventricular complex in the electrocardiogram without a corresponding arterial wave.

*Types of Waves during Effective Systoles.*—The different types of waves occurring during effective systoles are illustrated by the actual records shown in Figs. 2 to 9, but for a description, reference may be made to the transferred waves shown in the curves of Text-fig. 1.

The systolic waves may resemble typical impact waves such as are found in normal pulses. As in Type I, the rise is usually preceded by a short isometric period (A-B), during which vibrations of the first sound are discernible. The wave terminates in the vibrations of the second sound at C. The impact wave may, as shown in Type II, rise and fall sharply, which is also a frequent nor-

<sup>11</sup> Wiggers, *Arch. Int. Med.*, 1915, xv, 77.



TEXT-FIG. 1. I to VI represent different types of systolic waves occurring in auricular fibrillation. VII to X represent diastolic waves. Transcribed from the original curves and reduced about one-third.

Type I. Normal and intensified impact wave, often the only characteristic feature, and sometimes interpreted as the ventricular type of the venous pulse.

Type II. Peaked impact wave followed by systolic drop. It occurs when the tambour is pressed lightly and intravenous pressure is low during systole.

Type III. Intra-auricular type of venous pulse. It most nearly resembles the intra-auricular pressure curve and is only recorded when the arterial impact is feeble or absent.

Type IV. Double systolic waves both completed during systole. Probably due to tug or position change of contracting ventricle.

Type V. Systolic impact and stasis wave. Double systolic wave differing from Type IV in that the summit of the second wave terminated at the end of systole.

Type VI. Regurgitation wave due to tricuspid insufficiency.

mal variation (Fig. 1) when the tambour is very lightly applied. A direct impact may be almost or completely absent, as in Type III, in which a marked fall similar to that observed within the auricle, is the predominant feature. This is followed by a systolic rise (C-D), which also resembles the auricular pressure curve. This probably as nearly corresponds to a pure venous curve as it is possible to obtain and probably occurs only when the arterial waves are not vigorous enough to superimpose an arterial impact on the tambour record. A fourth type of wave is shown in Type IV where an impact oscillation (A-B-C) is followed by a second systolic oscillation (C-D-E). Its occurrence before the end of systole cannot be questioned, as the vibrations of the second sound occur after it terminates. Its significance cannot be fully explained. The possibilities that it may represent a tricuspid regurgitation late in systole, or an auricular stasis, do not entirely accord with the fact that it falls before diastole begins. The possible explanations to be considered are: (a) a ventricular position change which modifies auricular pressure and hence the jugular curve; and (b) a tug of the contracting ventricle on the superior vena cava. This is made more probable by the fact that waves of this type were particularly marked in two of our cases, both of which had definite signs of adhesive mediastino-pericarditis, and pericardial adhesions may well result in an unusual traction upon the superior attachment of the sac. A second type of double systolic wave is shown as Type V. This differs from Type IV in that the summit of the second wave terminates at the beginning of diastole.

What may be designated as regurgitation waves were also found. These waves have a distinctive contour—entirely different from the rounded ventricular waves described by Mackenzie and others in polygraph curves. These waves (Type VI) resemble waves found within the auricle when a relative insufficiency occurs during fibrillation of the auricles. Early in systole they rise sharply (A-B), then gradually rise further or remain sustained until the onset of diastole (C). Particularly characteristic are small undulations or vibrations superimposed on their summits which represent murmur vibrations transmitted to the supraclavicular region and generally audible there.

*Waves Occurring during Ineffective Systoles.*—During ineffective systoles the venous pulse may show nothing except a gradual stasis (Type VII) terminating at the end of systole; or the isometric period may be followed by a double vibration similar to, but smaller than those sometimes found in effective systoles (Type VIII). These waves may occasionally be shorter and more prominent (Type IX), and evidence of a small regurgitant wave is sometimes found (Type X).

#### *Diastolic Waves.*

The diastolic waves occurring during auricular fibrillation may be classed as large and small. The large oscillations are frequently found just before the systolic wave. In contour and duration they cannot be distinguished from normal presystolic (a) waves. Similar waves are also found during mid-diastole as shown at D in Fig. 9, and when this event is long they may occur in numbers. They apparently correspond to the waves found in Hewlett and Wilson's reported case. Instead of being a rare event, however, we find that they are a very common occurrence in photographically recorded venous pulses from cases of auricular fibrillation.

Three possible explanations are suggested for these waves, but it is not possible to decide between them on the basis of clinical experiment alone. It is conceivable that they may be produced by a coordinated mechanical contraction of a considerable portion of the auricular musculature which is sufficient to inaugurate a pressure wave within the auricle. According to this view, we must assume that the incoordinated fractionate contractions<sup>12</sup> of individual units of cardiac tissue happen to be so related that from time to time their interference produces an actual shortening of a certain section of the auricle. If this were the case we would expect simultaneous waves in the electrocardiogram which resemble normal P waves. Such waves are occasionally to be found, but the synchronism is by no means frequent.

It is possible that these waves indicate incoordinate coarse fibrillations of the auricle, as suggested by Hewlett and Wilson, since

<sup>12</sup> The term "fractionate contraction" has been defined by one of us as the interval that any unit of auricular tissue continues to shorten (*Am. J. Physiol.*, 1916, xl, 222).

they are irregular in size and bear no relation to smaller variations of the electrocardiogram. If this interpretation is true, then coarse fibrillations are far more common clinically than is generally believed.

Inefficient contractions of the ventricle might produce such waves. The possibility has been shown in animal experiments by one of us, and indeed was at the same time considered the most probable cause of these waves. Though virtually diastolic waves, they would really be due to unrecognized systoles. If this were true the electrocardiograms would show distinct ventricular complexes synchronous with them. A careful study of our records shows, however, that such inefficient systoles are rarely present in clinical fibrillation, and that very few such waves can be accounted for upon this basis.

The resemblance of the presystolic waves found in fibrillation to the second diastolic wave of the normal venous pulse has led Lewis<sup>13</sup> and others to attribute it, as has been done in the normal pulse, to a floating together of the tricuspid valves. Neither in the normal or fibrillating pulses does this interpretation appeal to us. The facts (a) that the wave is not associated with the third sound in cases where the latter has been recorded (Einthoven,<sup>14</sup> Eyster<sup>15</sup>), (b) that the valves do not come together sharply at the beginning of diastole, but apparently gradually float together throughout diastole (Dean<sup>16</sup>), (c) that, on dynamic principles, their closure is probably caused by a pressure difference rather than a wave of pressure within the auricle, all lead one to question such an origin in normal pulses. The fact that several such waves occur during long diastolic intervals in fibrillation would necessitate the assumption that the valves move into their position of closure several times, while an isolated presystolic wave occurring at the end of a long diastole would necessitate the assumption that in these beats the closure was, for some unaccountable reason, greatly delayed. That these waves are similar to the late diastolic waves normally found in long cycles cannot be denied; that either owes its origin to a valve movement seems improbable.

<sup>13</sup> Lewis, *Mechanism of the Heart Beat*, London, 1911, 204.

<sup>14</sup> Einthoven, W., *Arch. ges. Physiol.*, 1907, cxx, 31.

<sup>15</sup> Eyster, J. A. E., *J. Exp. Med.*, 1911, xiv, 594.

<sup>16</sup> Dean, A. L., Jr., *Am. J. Physiol.*, 1916, xl, 206.



They may be due to a position change of the relaxing or filling ventricle which exerts a pressure traction upon the auricles or large veins or perhaps causes a variation of intrathoracic pressure which might modify the influx and efflux of venous blood in the extra-thoracic veins.

### *Records of Representative Cases.*

As it is manifestly impossible to reproduce the many hundred yards of records taken from a total of 25 cases in the Second Medical Division of Bellevue Hospital, a description of a few short segments from representative cases must suffice for presentation. In this selection have been included (1) cases in which systolic waves predominate including (a) slow rhythms and (b) rapid rhythms, and (2) cases in which diastolic waves predominate (slow rhythms).

*Case H, 19.*—Male, aged 51 years. Rheumatic heart; no murmurs; compensating. Chronic nephritis. Systolic blood pressure, 165. (Fig. 2.)

The photographic record shows that systolic waves are predominant at the jugular region. Numerous attempts to obtain other venous waves of considerable size proved futile. Each systolic wave is nothing more than a transmitted arterial pulse. This is the characteristic record found in cases of auricular fibrillation accompanied by strong cardiac action and elevated blood pressure. The details of the central arterial pulse are readily discerned: 1-2, the initial vibrations; 3, the primary peak; and 4, the incisura with after vibrations. Their irregularity is the only conspicuous difference from normal arterial curves.

If we glance at the supraclavicular record taken by a Jacquet polygraph (Fig. 3) by way of comparison, it shows a series of systolic waves in every way typical of the ventricular type of venous pulse and referred to tricuspid regurgitation. It is clear that the deforming action of tambour levers may distort a perfectly normal arterial impact curve into the semblance of a regurgitation wave.

During the longer diastoles of Fig. 2 small waves occur (d-d) having a period of 0.076 to 0.128 of a second. Similar corresponding wavelets were also found in the apex curves.

*Case G, 6 and H, 8.*—Male, aged 44 years. Sclerotic heart; no murmurs; non-compensating. Chronic nephritis. Blood pressure, 220-150. (Fig. 4.)

This case showed in succession a variety of systolic wave types and in addition was characterized by numerous large diastolic waves during a certain period of his hospital stay. The cycles in this segment of record are numbered from 3 to 7 to facilitate description. Wave 3 represents a normal impact wave. The isometric period (a-b) is filled with fused preliminary vibrations after which the impact (b-c-d) occurs. Cycle 7 resembles this wave, but the systolic decline is sharper, placing it under Type II. Cycle 4 shows a bifurcated systolic wave (Type IV) which, to judge from the smaller amplitude of the corresponding radial beat, was produced by a less vigorous systole. Such smooth double vibrations were often found to accompany weak systoles in animal experiments previously reported by one of us.<sup>11</sup> Cycle 5 would probably have shown a similar double wave had not a weak systole occurred at 6 and cut short the second vibration. After the first elevation of Cycle 6, the curve continues to rise owing to the stasis of blood in the auricle which had not been allowed to empty because of the practical absence of preceding diastole. This double vibration due to an ineffective systole corresponds to Type IX.

During diastole a series of large waves ( $D_1$ ,  $D_2$ , and  $D_3$ ) occurs. While the third oscillation,  $D_3$ , is at its crest, Systole 4 occurs. Without a careful analysis of the interval of systole, these systolic waves following might be attributed to the same cause as the diastolic waves  $D_1$ ,  $D_2$ , and  $D_3$  and held to indicate a rhythmic coarse contraction of the auricle.

Attention should be called to the presystolic wave, P, which precedes Systole 7. In time and general appearance it resembles a normal presystolic wave, or a wave due to a coordinated auricular activity. These waves occur so frequently that to say presystolic waves are absent in auricular fibrillation compels one to ignore many such waves not to be distinguished from normal a waves.

To summarize, this record shows in rapid sequence a variety of systolic waves when ventricular activity is irregular in force, and the presence of large diastolic waves having a rate of 5.5 per second, or 330 per minute.

*Case H, 18.*—Male, aged 51 years. Sclerotic heart; rate 140–150 per minute; relative mitral insufficiency. General anasarca. Deep, somewhat dyspneic breathing. (Fig. 5.)

When the heart rate is rapid the venous pulse is composed almost entirely of systolic waves. When, in addition, breathing is deep, they are not only superimposed upon the respiratory variations, but their contour changes with inspiration and expiration, as in this record. During inspiration the individual waves are small and scarcely recognizable (*e.g.*, 2, 3, and 9). During expiration (Waves 1, 5, and 6) the waves are larger and have a distinctive contour which places them under Type II. The correspondence of the R peaks with each radial pulsation shows that each systole, even at that rapid rate, was effective.

*Case H, 17.*—Male, aged 35 years. Dilated, rheumatic heart; mitral regurgitation (Fig. 6); left-sided hypertrophy shown by electrocardiogram. A harsh systolic murmur was heard over the apex of the heart and in the right supraclavicular fossa.

The venous tracing shows a series of prominent regurgitant waves, the characteristic features of which are a rapid rise during the onset of systole ending in a sustained ascending or descending plateau upon which a series of vibrations, probably resulting from the concurring murmur, are superimposed. In contour they correspond to intra-auricular waves found in experimental animals which have tricuspid regurgitation. The onset of systole as shown by the R wave of the accompanying electrocardiogram (not reproduced) is marked at S in each case. It is apparent that their exact contour depends to some extent upon the state of venous pressure at the onset of systole. Beat I represents a typical unaltered regurgitation curve. In Waves IV and V the onset of systole occurred at the summit of a diastolic stasis rise (S) and consequently the main rise of the wave occurred before the onset of systole. This is a frequent occurrence when the heart rate is rapid.

*Case H, 11.*—Male, aged 40 years. Rheumatic heart; very large. Systolic murmur over aortic area transmitted to the neck; diastolic murmur over sternum transmitted to the apex; systolic murmur at the apex transmitted to the axilla (Fig. 7).



The record of Fig. 7 was taken on February 19, 1915. The pulse rate averaged 150 per minute. The venous pulse is chiefly made up of waves indicating a dilated right heart. Two types are prominent. Those marked Type V show after a primary systole wave, which is probably an impact, a considerable stasis rise. Several waves, owing to weaker systoles, show little of the first impact wave and consist essentially of the early systolic fall and subsequent rise. These are the waves that resemble the intra-auricular pressure curves (Type III). Between these waves occur typical regurgitant waves, which rise rapidly and remain sustained with numerous vibrations superimposed throughout systole (Type VI).

Another record shown as Fig. 8 was taken on March 1, 1915, after rest and digitalis treatment had slowed the pulse to an average of 86 per minute. This lengthened the diastolic interval which is consequently filled with many diastolic waves (d) varying considerably in amplitude and period. There are no corresponding waves found in the electrocardiogram record. Evidence of regurgitation waves is practically absent.

*Case H, 1.*—Male, aged 38 years. Infective endocarditis and mediastino-pericarditis; double mitral lesion; coarse systolic thrill over precordium (Fig. 9). Upper curve, supraclavicular venous pulse; lower curve, apex tracing.

During systole two distinct waves occur in the apex tracing, one (1-2-3) during the early half of systole, the second (3-4-5) during the latter half. Superimposed upon these waves occurs a series of finer waves extending throughout systole, as can be seen from the irregular width of the ascending line (3-4-5). They represent the first sound vibrations together with the systolic murmur. Systole ends with the few vibrations at S<sup>2</sup>.

Each systole produces in the jugular pulse two waves, 1-2-3 and 3-4-5, which, allowing for delay, correspond exactly to the apex waves. The probability that this double systolic wave (Type IV) is due to a position change or tug of the ventricle has been previously discussed.

Early in diastole of each cycle occurs a wave D similar to that sometimes found in the normal curve. When the cycles are long this wave may precede systole and simulate a presystolic wave, as in the third cycle shown.

TABLE I.

*Relative Frequency of Different Types of Waves.*

Case No.	Age.	Clinical diagnosis.	Systolic waves.					Diastolic waves.
			Normal impacts. Types I and II.	Intra-auricular curves, feeble ventricular action. Type III.	Systolic tug or position change. Type IV.	Stasis curves. Type V.	Regurgitation curves. Type VI.	
G, 1	32	Rheumatic heart; mitral stenosis.	All.					Few fine in long diastoles. Period short.
G, 2	21	Rheumatic heart; mitral regurgitation.					All.	Many coarse. Period irregular.
G, 5	62	Sclerotic heart; relative mitral regurgitation.				All.		Few coarse.
G, 6-H, 8	44	Sclerotic heart; no murmurs; chronic nephritis. Blood pressure 220-150.	Many.		Few.			Many coarse. Frequency, 330 per min.
G, 7	54	Rheumatic heart; double mitral lesion.	All.					None.
G, 9	26	Rheumatic heart; double mitral lesion.	Many.			Few.		Many fine in long diastoles. About 750 per min.
G, 10	42	Rheumatic heart; mitral regurgitation.			All.			None.
G, 11	38	Rheumatic heart; mitral regurgitation.	All.					Many coarse.
G, 14	26	Rheumatic heart; mitral regurgitation.	"					None.
H, 1	38	Infective endocarditis and mediastino-pericarditis; double mitral lesion.	Many.		Many.			Many fine. Frequency 460 per min.
H, 5	48	Postpneumonic mediastino-pericarditis; dilated heart; no murmurs.	"		"			Occasional presystolic.
H, 7	61	Sclerotic heart; dilated; no murmurs.	All.					Many fine.
H, 9	36	Rheumatic heart; mitral regurgitation.	"					None.
H, 10	30	<i>Streptococcus viridans</i> polyserositis; dilated heart; relative mitral regurgitation.	"					Occasional coarse. Period from 0.076 to 0.128 sec.

TABLE I—*Continued.*

Case No.	Age.	Clinical diagnosis.	Systolic waves.					Diastolic waves.
			Normal impacts. Types I and II.	Intra-auricular curves, feeble ventricular action. Type III.	Systolic tug or position change. Type IV.	Stasis curves. Type V.	Regurgitation curves. Type VI.	
	<i>yrs.</i>							
11	40	Rheumatic heart; mitral regurgitation; aortic regurgitation and roughening.	Few.	Few.		Many.	Many.	Few coarse. Frequency, 300 per min.
12	48	Rheumatic heart; double mitral lesion.	All.					Few presystolic.
13	37	Rheumatic heart; mitral regurgitation.	"					Many fine.
15	27	Rheumatic heart; double mitral lesion.	Many.			Few.	Few.	" " Frequency, 450 per min.
16	24	Rheumatic heart; double mitral lesion; Graves' disease.	"			"		Few fine. Period from 0.07 to 0.10 sec.
17	35	Rheumatic heart; mitral regurgitation.	"				Many.	Few fine.
18	51	Sclerotic heart; relative mitral regurgitation.	All.					None.
19	51	Rheumatic heart; no murmurs; chronic nephritis.	Many.			"		Few coarse in long diastoles; more as ventricle slowed. Period from 0.096 to 0.128 sec.
20	38	Rheumatic heart; double mitral lesion.	All.					Few fine.
21	55	Sclerotic heart; relative mitral regurgitation.	Few.			"	"	" "
23	34	Rheumatic heart; double mitral lesion.	"	Many.		"		Many fine. Period from 0.066 to 0.116 sec.

In the apex curve as well as the jugular curve occur small wavelets (d-d) whose period averages 0.13 to 1.1 seconds. Their rate averages about 460 per minute.

This case, illustrating a fibrillating auricle with slow ventricular

rhythm, shows in the jugular a typical double systolic wave, a normal diastolic variation, and, in addition, small periodic diastolic waves.

The electrocardiogram showed similar diastolic waves of considerable amplitude, which did not correspond definitely with the waves in the phlebogram.

The relative frequency with which the various types of waves occurred in our cases is shown in Table I.

By far the most frequent form of systolic wave is the normal impact wave (Types I and II), which was present in all but three cases. They often constitute the most conspicuous, and in certain cases (twelve of our series) the only characteristic feature of the venous pulse curves. They may resemble each other from one cycle to another, which is usually the case when the heart is slow and comparatively regular, or they may show rhythmical variations in size with respiration, especially when the heart is rapid yet fairly regular, or when moderate dyspnea is present. They may vary from beat to beat both in contour and amplitude, these being determined, as has been previously analyzed by one of us,<sup>11</sup> by (*a*) the vigor of ventricular systole, (*b*) the height of aortic pressure at the onset of systole, (*c*) the duration of the previous diastolic interval, and (*d*) the pressure of the recording tambour.

When the cardiac contraction is particularly vigorous the systolic wave may resemble a typical central arterial pulse terminating in a deep incisure. The important observation was made that when this occurs the venous pulse recorded by the polygraph is unusually distorted and often resembles the ventricular waves of Mackenzie, which are supposed to indicate regurgitation. So called ventricular waves of polygraph curves may thus be produced by perfectly normal arterial impacts and are not indicative of tricuspid regurgitation; on the contrary, they may signify exceptional heart action.

Next in frequency is the stasis curve (Type V), being found in eight cases. In only one instance were they the only curves present, and, as might be anticipated, they were most frequent when the heart was incompetent and venous stasis was evident in the pulmonary or portal circulations.

The type of wave which we attribute to a systolic tug or position change of the heart (Type IV) was present in four cases. In two of

these cases well marked signs of pericardial adhesions were noted, while in another case though no physical signs of the condition were recorded, the rheumatic origin of the heart lesions makes its presence not improbable. In the fourth instance pericardial adhesions were not suspected.

Intra-auricular pressure waves (Type III) were present in only two cases. Regurgitation waves (Type VI) were present in five cases, and in one of these they disappeared during the course of hospital observation.

This observation discredits the idea that tricuspid regurgitation is a frequent accompaniment of auricular fibrillation.

#### SUMMARY.

While auricular fibrillation is easily recognized by arterial and jugular tracings or by electrocardiograms, it is not possible to interpret all the waves found in these records.

The venous tracings may in general be placed in one of two classes: (a) those in which prominent systolic waves predominate or occur alone; and (b) those in which large or small diastolic waves occur occasionally, in groups, or in a continued series averaging 250 to 500 per minute. The prominent systolic waves have generally been attributed to tricuspid regurgitation, the inference being that tricuspid regurgitation is a common state in auricular fibrillation. The diastolic waves are so numerous and so many electrocardiograms show diastolic waves that it is impossible to account for them on the assumption that the auricle is in a dilated and finely fibrillating state. It has been suggested that in these instances a condition of coarse fibrillation, which is closely allied to auricular flutter, obtains. The systolic and diastolic waves of the venous pulse of twenty-five clinical cases of auricular fibrillation, recorded by photographic methods, were studied.

Six types of systolic waves (Text-Fig. 1) were found: (1) an intensified impact wave, the most common and often the only characteristic feature, indicating vigorous ventricular action; (2) a peaked impact followed by a rapid systolic drop due to light pressure of the tambour; (3) the intra-auricular type of systolic variation, so called from its resemblance to intra-auricular pressure



curves found in animals, occurring in clinical cases only when ventricular systole is weak; (4) double systolic waves, attributed to a systolic tug of the ventricle on the auricles and large veins; (5) a systolic impact followed by a stasis wave, present when intravenous pressure is high; (6) a regurgitation wave composed of a steep rise continued into a systolic plateau with murmur vibrations superimposed.

Our study showed (1) that tricuspid regurgitation, as indicated by the presence of regurgitation waves, is a rare accompaniment of auricular fibrillation; and (2) that the contrary opinion, arrived at by the frequent presence in polygraph tracings of ventricular types of waves, is due to the fact that the contour of intensified impact waves is distorted by polygraph levers so that they simulate regurgitation waves.

Recurrent diastolic waves were frequently present in our records. Their relative size depended, to a considerable extent, on the pressure of the tambour. There was no constant relation to similar waves in the recorded electrocardiogram, nor is it proven that they are indicative of a coarse type of fibrillation or an associated flutter.

#### EXPLANATION OF PLATES.

##### PLATE 1.

FIG. 1. Curve showing the character of the normal apex and venous pulses photographically recorded. Reduced nine-tenths.

FIG. 2. Curves of supraclavicular and radial pulses from a case of auricular fibrillation in which prominent arterial impact waves predominate. During diastole small waves (d-d) sometimes recurred. Reduced six-tenths.

FIG. 3. Venous tracing taken by a Jacquet polygraph from the same case as Fig. 2, showing the so called ventricular type of the venous pulse.

##### PLATE 2.

FIG. 4. Curves of supraclavicular and radial pulses from a case of auricular fibrillation showing the variety of systolic waves and numerous large diastolic waves. Reduced eight-tenths.

FIG. 5. Supraclavicular and radial pulses in auricular fibrillation with rapid heart rate and deep breathing. The lines correspond to the rise of the R waves of the electrocardiogram simultaneously recorded but not reproduced. Reduced nine-tenths.

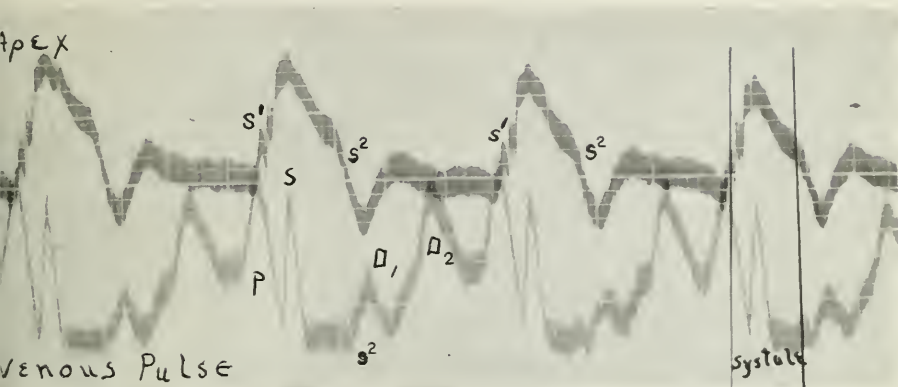


FIG. 1.

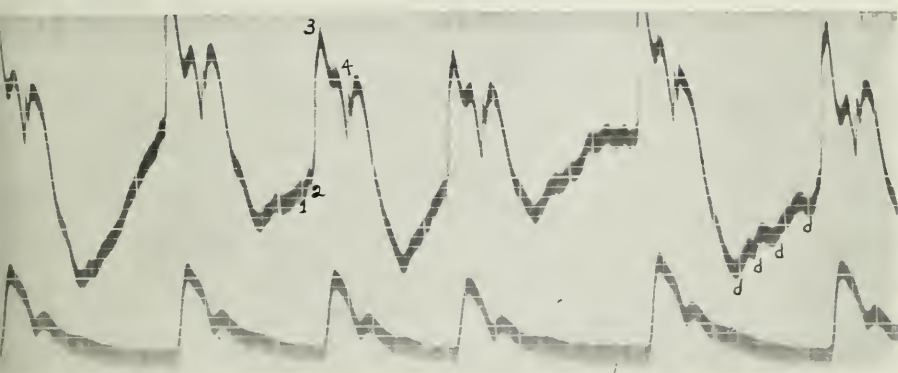


FIG. 2.

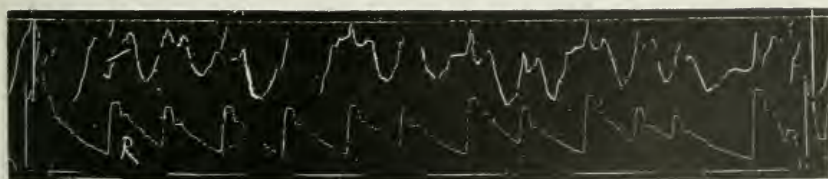


FIG. 3.

(Niles and Wiggers: Venous Pulse in Auricular Fibrillation.)





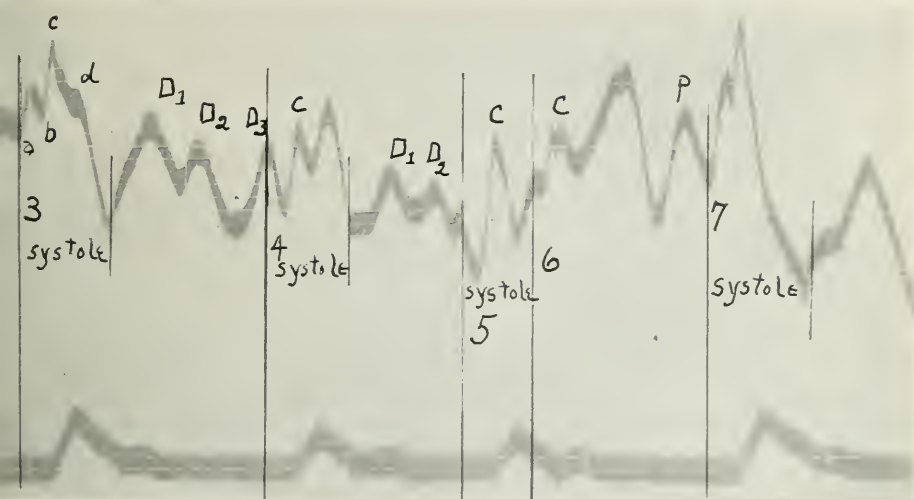


FIG. 4.

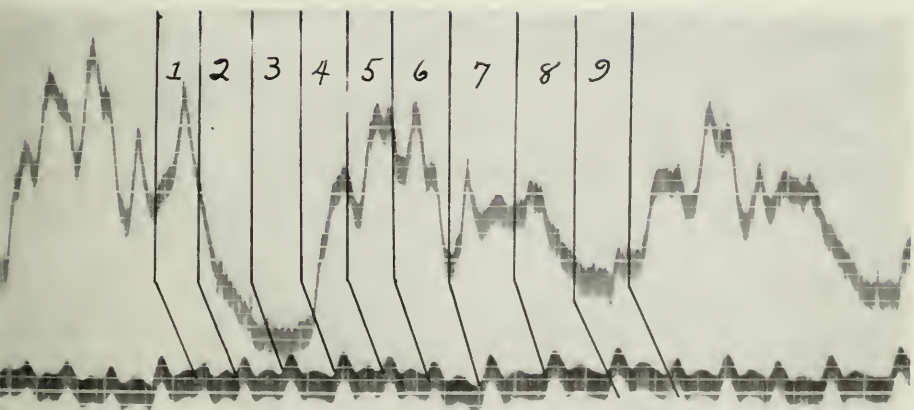


FIG. 5.

(Niles and Wiggers: Venous Pulse in Auricular Fibrillation.)



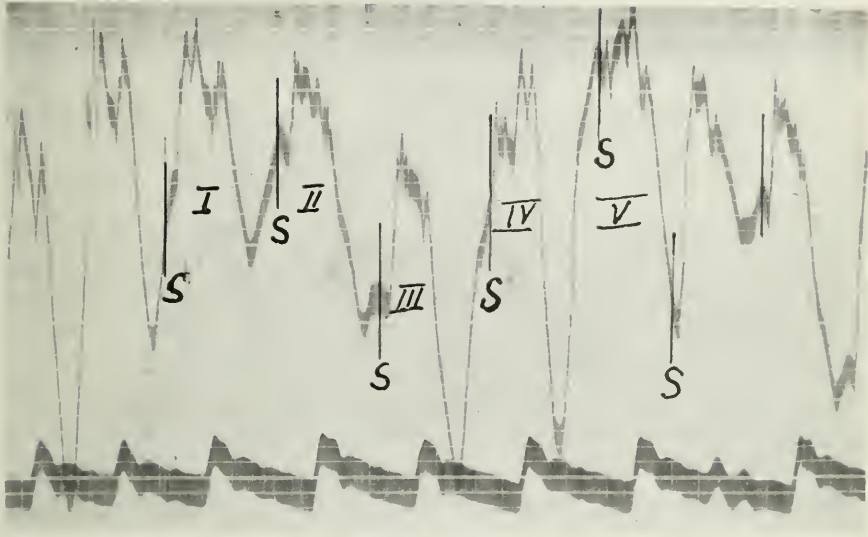


FIG. 6.

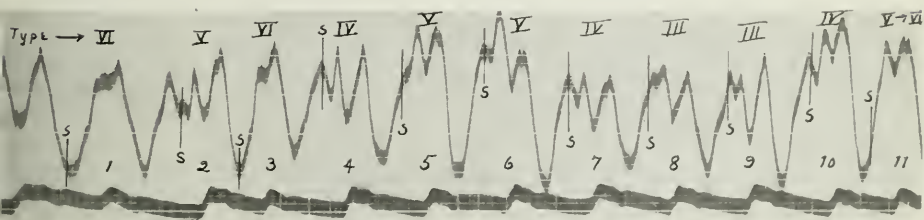


FIG. 7.

(Niles and Wiggers: Venous Pulse in Auricular Fibrillation )



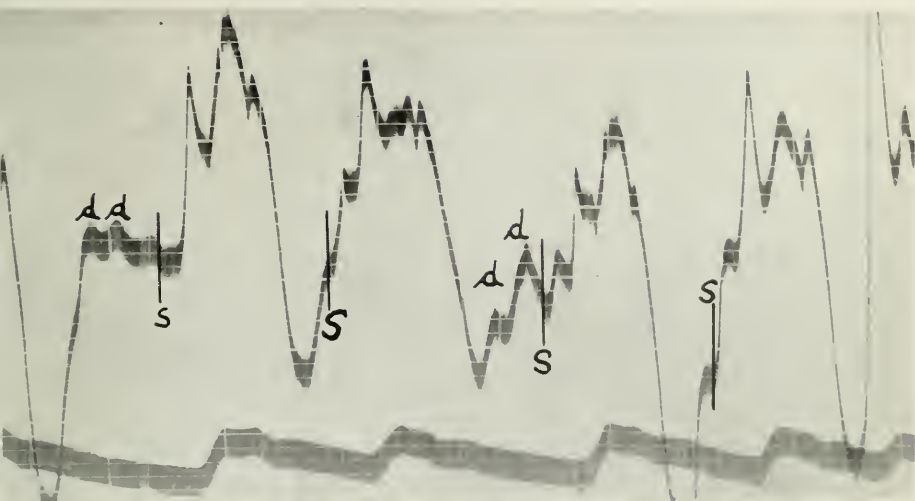


FIG. 8.

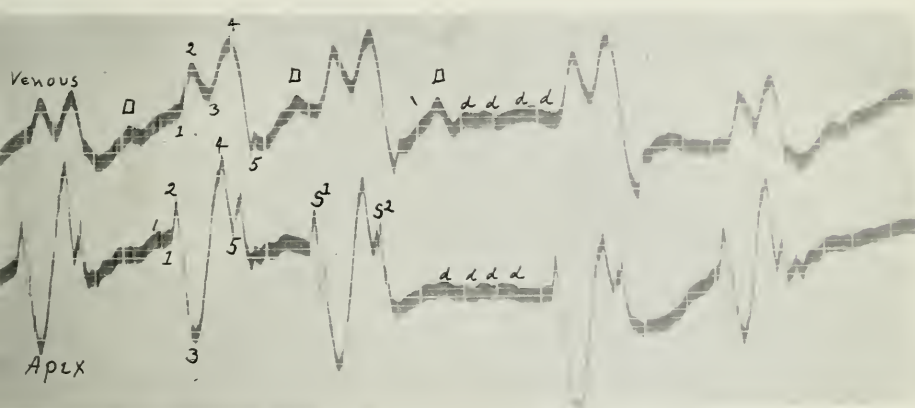


FIG. 9.

(Niles and Wiggers: Venous Pulse in Auricular Fibrillation )



## PLATE 3.

FIG. 6. Supraclavicular and radial pulses in auricular fibrillation. The former shows regurgitant waves almost exclusively. Lines are drawn to correspond to R waves of the electrocardiogram simultaneously recorded, but not reproduced.

FIG. 7. Supraclavicular and radial pulses from a case showing a gradation of systolic types of waves. Marked according to the scheme shown in Text-fig. 1. Reduced one-half.

## PLATE 4.

FIG. 8. Venous and radial pulses from the same case as Fig. 7 after rest and digitalis medication. The absence of regurgitation waves and the presence of diastolic waves is shown. Reduced nine-tenths.

FIG. 9. Venous and apex curves, showing similarity of waves (p. 13). Reduced about one-half.





# THE SIGNIFICANCE OF THE DIASTOLIC WAVES OF THE VENOUS PULSE IN AURICULAR FIBRILLATION.

BY CARL J. WIGGERS, M.D., AND WALTER L. NILES, M.D.

(From the Physiological Laboratory and the Department of Medicine of Cornell  
University Medical College, New York City.)

PLATES 5 TO 7.

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## *General Outline of Plan of Study and Results.*

In a previous study of the photographic venous tracings taken from clinical cases of fibrillation,<sup>1</sup> it was shown that the types of waves occurring during systole could be satisfactorily harmonized with and explained by photographic tracings previously obtained from animal experiments. The diastolic waves, however, could be given several different interpretations. This phase of the subject was, therefore, submitted to further experimental investigation.

The plan of study resolved itself into several phases. In the first place, it was desirable to determine whether large diastolic waves in the jugular tracings and in the electrocardiograms of many cases are diagnostic of a state of coarse fibrillation or associated flutter, or whether they are also present during fine fibrillation. Anticipating somewhat the results presented later, it was found that although coarse fibrillation was accompanied by somewhat larger diastolic waves, vibrations of considerable amplitude could be present during a state of fine fibrillation. In the electrocardiograms the diastolic waves were sometimes larger during the state of fine fibrillation.

The second phase of the experiments concerned itself with the cause of these diastolic variations. It at once became important to establish whether they represented pressure variations from the right auricle. To determine this, the right intra-auricular pressure was

<sup>1</sup> Niles, W. L., and Wiggers, C. J., *J. Exp. Med.*, 1917, xxv, 1.

recorded by introducing into the auricle by way of the azygos veins and superior vena cava, a sensitive venous optical manometer (Wiggers<sup>2</sup>). Again anticipating, it was found that with very few exceptions the true diastolic waves<sup>3</sup> were not accompanied by similar variations of intra-auricular pressure, even when extremely sensitive manometers were used.

By elimination it is impossible to ascribe the jugular waves to any other factor than a traction of some portion of the heart upon the veins leading to the lower cervical region. By attaching threads connected with delicate tambour systems to different portions of the veins, and by studying the accurately recorded myograms of the auricle, it was found that during fibrillation a distinct periodic traction is exerted upon the veins and that this corresponds in rhythm with the diastolic waves of the venous pulse.

What type of cardiac action is able to affect the extrathoracic veins so as to alter their lumen and pressure without acting upon the intra-auricular pressure? Previous experimentation had shown that position changes of the contracting and relaxing ventricles are capable of exerting a traction upon the auricle. A comparison of these traction oscillations of the auricular myogram with the traction curves of the large veins and with the venous waves, led to the conclusion that the position changes of the relaxing ventricle could produce the large oscillations, which accompany fine fibrillation. This, however, is not the only mechanism capable of producing diastolic waves during coarse fibrillation. It was shown by myographic curves and auricular electrograms that the irregularly spreading excitation waves strike different areas of the auricle in such a way that they undergo irregular mechanical contractions. These contractions are apparently also able to exert a traction on the veins sufficient to cause diastolic waves in the venous pulse. These coarse contractions bear no relation to the electrical waves and the latter are therefore not of differential value between coarse and fine fibrillation.

<sup>2</sup> Wiggers, C. J., *Arch. Int. Med.*, 1915, xv, 77.

<sup>3</sup> Double rounded waves, which are systolic in time, may, as previously pointed out, be mistaken for diastolic, unless accurate evidence of the presence of a systole is recorded. Such waves are accompanied by intra-auricular pressure variations.

Having thus outlined the plan and object of the experiments and anticipated the results in a general way, it is desirable to present more in detail the precise procedures and results from which the conclusions are derived.

### *Experimental Procedures.*

Dogs anesthetized with morphine and chloretone were used. The external jugular vein was dissected low down in the neck. Over the venous pulsation a small tambour (2 cm. in diameter) covered with light rubber was lightly pressed and the volume changes of the vein were communicated to Frank's segment capsules as in clinical cases. Electrocardiograms were recorded from the right fore leg and left hind leg (Lead II) by Dr. Williams' American model of Einthoven's string galvanometer.

The thorax was opened and records of the intra-auricular pressure were taken by the type of optical manometer described elsewhere (Wiggers<sup>2</sup>). This instrument was, in a number of experiments, made exceedingly sensitive with the hope of detecting the slightest variation of intra-auricular pressure. So used, these manometers unfortunately pick up vibrations of the animal and of the building which appear as small rhythmic oscillations, and must, of course, be discounted in the analysis of records.

To various points on the right auricle were stitched several miniature myocardiographs recently described (Wiggers<sup>4</sup>). These instruments recorded, through optical capsules, the approximation and recession of the points to which they were attached.

Auricular fibrillation was induced by applying a moderate tetanizing current by a pair of ordinary platinum electrodes, or by sending the current through pin electrodes inserted into widely separated points on the auricle. The latter mode of stimulation more frequently produced a fine fibrillary movement; the former, a coarser fibrillation. We also attempted to produce fine fibrillation by simultaneously stimulating the right vagus, as reported by Robinson.<sup>5</sup>

When an electrocardiogram was being taken, the electrical variations of the stimulating current naturally spread so as to affect the

<sup>4</sup> Wiggers, *Am. J. Physiol.*, 1916, xl, 218.

<sup>5</sup> Robinson, G. C., *J. Exp. Med.*, 1913, xvii, 429.

galvanometer leads and hence are indicated in these records. For this reason the fibrillating movements persisting immediately after cessation of stimulation were recorded whenever possible.

During fibrillation of the auricle the ventricular rhythm becomes irregular and the rate exceedingly rapid. This condition does not correspond to the majority of cases seen clinically after treatment where the beat is slow. A corresponding slowing was experimentally produced in a number of ways, the two most successful means of producing a prompt slowing being the stimulation of the left vagus and the use of pituitrin.

### *Analysis of Results.*

Anyone who is familiar with the technical limitations of optical experiments realizes that it is not feasible to record more than three or four circulatory records at a single time. Hence various combinations of tracings were taken in a total of 18 experiments. In this way the different events were related and the results many times duplicated. In analyzing the results it is not possible to proceed in a manner as methodical as that used in the general outline of results, because each record brings out isolated points which make the basis of the facts. It is proposed, however, in the selected records appended to discuss each phase of the investigation as outlined.

Fig. 1 is a section of a record taken on June 16, 1916. The intra-auricular pressure, the venous pulse, and auricular myogram from two points on the auricle 16 mm. distant were recorded. They are indicated on the record.

The record starts while the auricle is in fine fibrillation caused by the continued passage of a moderate tetanizing current. This condition continued during the period marked A-B. Upon cessation of stimulation the auricle lapsed into a state of coarse fibrillation, extending through the interval marked B-C. This was followed by a condition of regular auricular and ventricular beats.

The myogram record is interesting, first as showing the mode of cessation of auricular fibrillation in the dog. It is at once apparent that fine fibrillation (A-B) causes exceedingly small variations in the myogram curve, which cannot be recorded at all by ponderable

levers. This means that the combined effects of the separate fibrillary movements spreading irregularly over the auricle do not by interference or summation act to produce a mechanical shortening of auricular tissue. When stimulation is discontinued the auricle of dogs frequently regains its normal rhythm rapidly. Before this occurs, however, the auricle lapses into a condition of coarse fibrillation. During this interval (B-C) the auricular myogram shows definite waves indicating that distinct mechanical shortening is taking place. Within the interval of 1 second six such waves occur, making their rate 360 per minute. During this period, moreover, two large beats (M, N) occur which resemble the myograms of coordinated contractions. An examination of the intra-auricular pressure curve, taken with a very sensitive manometer (1 mm. = 0.8 mm. saline), shows that after discounting the fine periodic vibrations (1, 2, 3) due to extraneous vibrations and the waves due to ventricular systole (sys), there are no waves during either fine or coarse fibrillation. The first auricular attempt at a definite contraction (M) is also accompanied by no marked rise of intra-auricular pressure. The second contraction (N), on the contrary, is accompanied by a definite wave (N'). Time comparisons show that the rise of pressure corresponds absolutely to the onset of the myogram contraction. The onset of real coordinated contractions (O, P) is indicated by their characteristic contour (Wiggers<sup>4</sup>). These contractions are preceded for the first time by the pressure rise, as is normally the case.

The second point of interest lies in the relation of the diastolic waves of the venous pulse to the auricular myogram and intra-auricular pressure variations. It will be seen that between the waves accompanying ventricular systole (sys) are definite diastolic waves (a) occurring at the rate of 6 to 8 per second. Although somewhat smaller, they are present during coarse as well as fine fibrillation. During the former condition they appear without any evidence of mechanical shortening in the auricular myogram. During the latter condition, as shown by the lines sketched, they follow a short interval after the smaller oscillations in the myogram.

A careful consideration of the records brings out the following points. (1) When the auricle passes out of a condition of fine fibrillation, it first passes through a stage of coarse fibrillation. During



this stage definite mechanical contractions gradually appear and these finally give way to normal coordinated contractions. (2) Diastolic waves of the venous pulse may occur during the state of fine as well as coarse fibrillation. In the latter condition they are, however, generally larger in amplitude, and correspond to an actual mechanical shortening of the auricle. (3) The diastolic waves of the venous pulse are not due to transmitted auricular pressure variations during fibrillation. Such variations occur only when the auricle gives coordinated contractions.

The records shown as Fig. 2 are from an experiment on March 8, 1916. The upper record shows the auricular myogram taken by a miniature myocardiograph and recorded by a segment capsule more sensitive than that used in the records of Fig. 1. The electrocardiogram (Lead II) is recorded below with time divisions of 0.05 of a second.

The records are typical as showing the relations between the electrical variations and accurately recorded mechanical changes in the auricle during fine and coarse fibrillation. The record starts with the auricle in fine fibrillation, a condition produced in this instance through simultaneous stimulation of the right vagus. When vagal stimulation was stopped the auricle gave several large contractions (1, 2, 3) simulating coordinated beats, after which it lapsed into a coarse fibrillation.

To facilitate reference to specific waves, the ventricular systoles as indicated by the R peaks in the electrocardiogram and the corresponding sound vibrations in the myogram are marked alphabetically. Following each R peak in the electrocardiogram a wave corresponding in time and contour to a T wave must be recognized.

During fine fibrillation the auricular myogram clearly shows a series of small mechanical variations irregular in period and amplitude. The electrocardiogram also shows small rhythmic variations. They are only occasionally related to the mechanical shortening. Thus between Systoles A and B only eleven distinct mechanical variations occur while in the electrocardiogram at least sixteen waves are present. Of the mechanical variations two ( $x'$ ) are definitely preceded by electrical waves( $x$ ); but two other distinct mechanical waves ( $w, y$ ) are entirely without corresponding electrical waves.

Toward the end of the record where coarse fibrillation had taken place the auricular myogram shows a series of larger but still irregular mechanical contractions. If we eliminate the variations of the electrocardiogram which probably represent T waves, there remain very few waves that correspond to the mechanical contractions.

Separating the interval of coarse and fine fibrillation is an interval where several coarse beats occur (1, 2, 3) which somewhat resemble coordinated beats. A comparison of the electrical variations for these beats indicates that Wave 1 is possibly preceded by a corresponding electrical variation; Wave 2 is certainly preceded by no such wave, while it is impossible to determine whether the electrical variation accompanying Wave 3 represents a T wave or an auricular wave.

A study of the curves makes it evident (1) that while large electrical variations may be present during coarse fibrillation they are by no means a constant accompaniment or diagnostic of this condition, (2) that distinct oscillations in the electrocardiogram more frequently accompany fine fibrillation, and (3) that the lack of relation between mechanical and electrical variations during ventricular diastole are characteristic of auricular fibrillation.

Fig. 3, taken on June 25, 1915, shows in order, the external jugular pulse, the auricular myogram, and the intra-auricular pressure curve. The auricles were in a state of coarse fibrillation at the beginning of the record. Then the left vagus was stimulated, resulting in a state of finer fibrillation together with a slower ventricular rhythm.

The curves are of interest as showing first, the influence of mechanical auricular movements on the jugular and venous pressure during long diastoles. The case duplicates the clinical cases slowed by digitalis medication. The beginning of each ventricular systole is indicated by a series of large vibrations in each record (A). The end of systole can be determined approximately by the fall of intra-auricular pressure (B). During the first long diastolic period (1-2) the auricular myogram contains only very small variations, and except for an expected gradual rise of pressure the intra-auricular curve shows no oscillations. Each ventricular systole, however, exerts a definite traction on the auricle consisting of a primary vibration (a-b-c-d) and a slower secondary movement (d-e-f). It will be



noticed that waves corresponding to these tractions are communicated to the jugular vein, and are shown in the upper record. This indicates that ventricular systole, which in the normal heart exerts some traction upon the auricle, pulls even more vigorously upon the distended auricle and veins during fibrillation. Early in diastole a decremental series of oscillations occurs in the auricular myogram (g-h-i-j-k). As similar waves occur in long normal cycles, they can only be interpreted as due to position changes of the relaxing ventricle in both cases. It is evident that these waves are sometimes accompanied by waves in the venous pulse.

Experiments of this nature show that: (1) When diastole is long few wavelets in the myogram or in the jugular can be ascribed to the fibrillating auricle itself. (2) Both ventricular systole and diastole by active traction and recession are capable of producing a passive shortening and lengthening of the auricular fibers, and these may produce similar effects on the veins. This traction communicated to the veins is at least one cause of the large diastolic waves occurring during fibrillation.

Fig. 4 is another record taken from the same animal. It shows a state of coarse fibrillation modified by the action of pituitrin, which moderately slowed the ventricle.

The jugular tracing shows a series of rhythmical waves averaging 300 per minute. From such a record the erroneous impression could easily be derived that the auricles were contracting at this rate. A comparison with other records shows that the auricular mechanical contractions were entirely irregular and that the jugular waves were the result of a fortunate interference between the effects of auricular beats and ventricular traction. Upon the waves are superimposed smaller vibrations which upon careful study can be assigned only to heart sound vibrations. Such tracings show that large coarse rhythmical waves in the jugular must be guardedly accepted as evidence of regular auricular activity.

Fig. 5 shows four segments of a tracing taken on March 8, 1916. The upper curve is a record of the movements of the superior vena cava; the second curve is an auricular myogram; while the lower record is an electrogram obtained by leading off from the two auricular points to which the myogram was attached.

Segment A shows a normal cycle obtained during stimulation of the right vagus. The auricular myogram has the contour described by one of us (Wiggers<sup>4</sup>) as characteristic for a coordinated beat. Ventricular systole, the duration of which is indicated by the two sound vibrations (1S-2S) in the superior vena cava record, causes a jog (a) and several secondary waves (a-b-c-d) on the relaxation curve of the myogram. During early ventricular diastole another wave (d-e-f) is added. These tugs are transmitted to the wall of the superior vena cava as shown in the upper record. Slightly preceding the auricular myogram, the auricular electrogram shows a diphasic variation (1-3) evidently of auricular origin. This is followed by an R peak derived from ventricular activity. As no electrical variations accompany the secondary waves of the myogram (a-b-c-d-e-f) they cannot be assigned to an active contraction, but must be due to passive traction.

Segment B was taken during a state of auricular tachyrythmia (flutter) induced by previous tetanic stimulation of near points on the right auricle. Each ventricular systole is indicated by a small positive peak (R) in the electrocardiogram and by a series of sound wavelets (S) in the superior vena cava record. In contradistinction to the secondary wavelets following a normal vagus beat, for example, each mechanical variation in the myogram is accompanied first by a small positive, then by a large negative variation in the electrogram. The incidence of irregularly recurring ventricular systoles results in irregular traction upon the auricle and hence the auricular myogram waves during flutter appear of irregular amplitude.

Segment C shows the transition of an existing auricular flutter to a coarse fibrillation upon stimulating the right vagus. The myogram as well as the superior vena cava record still shows a series of small waves which could not have resulted from a ventricular traction, for (1) complete stoppage of the ventricles had occurred, and (2) the electrogram shows a rapid series of irregular variations.

Segment D shows a portion of the curve after vagus stimulation had ceased. The auricle lapsed into a state of coarser fibrillation and the ventricle resumed its beat. Owing to the large auricular waves present in the electrogram derived directly from the auricle, the ventricular systoles are difficult to differentiate in the electrogram,

but are clearly marked by sounds (S) in the superior vena cava record. While it is probable that some of the mechanical variations of the myogram are due to the traction of these ventricular systoles, the presence of the numerous electrical variations of undoubted auricular origin shows that many of the mechanical variations must have been due to the coarse fibrillation of the auricle itself.

A study of these records shows that: (1) Ventricular position changes may cause a series of passive mechanical changes in the auricle (unaccompanied by an action current) which may be the source of some diastolic variation in the venous system. (2) Since the waves during auricular flutter and coarse fibrillation are accompanied by electrical variations and since these waves may occur when ventricular activity is entirely in abeyance, it follows that not all the mechanical variations in the myogram and superior vena cava can be accounted for by ventricular traction. On the contrary, it must be assumed that during coarse fibrillation the impulses travel irregularly over the auricle and excite the various sections of auricular musculature to irregular active contractions, and that these contractions by drawing upon the veins cause large oscillations to appear in the venous pulse.

#### SUMMARY.

With the clinical recognition that different degrees of fibrillation occur and that these in turn are closely related to a coordinated type of auricular tachyrhythmia (flutter); further, that one type may lapse into another or into a perfectly normal rhythm, the conviction has grown that finer and coarser types of auricular movement may be recognized by the amplitude of the diastolic waves of the electrocardiogram and venous pulse. The present investigation into the cause of these waves has shown that this is not possible. There is no theoretical or experimental reason for the assumption that any fixed relation exists between the amplitude of the electrical variations of the electrocardiogram, which are the resultant of variations accompanying the irregularly spreading excitation wave, and the degree of mass contraction following. The large recurrent waves of the venous pulse, which may with more reason be regarded as related to the size of the auricular mechanical contractions on theoretical

grounds, are also shown to be without differential value. The reasons for this may be briefly summarized.

In the first place, the presence of diastolic waves is contingent upon a slow heart rate and long ventricular diastoles. With rapid heart rate their occurrence is prevented by the closely placed systolic variations. It is therefore conceivable that both coarse and fine fibrillation as well as flutter will be without diastolic waves as long as the heart is rapid.

Even in the cases with long beats present their significance must remain doubtful. It is true that in the experiments the waves accompanying coarse fibrillation are as a rule somewhat larger than those occurring during fine fibrillation; waves of considerable size may, however, be present in fine fibrillation. Hence, as long as no calibrated method of recording is possible, it is difficult to draw any inference. It is possible from the same patient to record with the same apparatus diastolic waves of varying amplitude by merely changing the pressure of the receiving tambour.

The chief reason that the amplitude of these waves cannot be regarded as of differential value is found in their origin. Fine fibrillating movements of the auricle do not in themselves produce waves in the jugular. They produce neither pressure variations in the auricle, nor exert any traction upon the veins. The only factor capable of producing jugular waves during fine fibrillation seems to be the traction exerted by the position changes of the ventricle on the auricle and large veins. This may, in a measure, explain why the diastolic waves recorded from the apex and second left interspace of patients often closely correspond with those simultaneously recorded from the jugular. The term "fibrillary waves" commonly applied to the smaller of these variations is evidently poorly chosen when their etiology is considered. The coarser contractions of the auricle during coarse fibrillation also produce no pressure changes within the auricle. They are vigorous enough at times, however, to exert a traction upon the venous walls. Hence, the waves during coarse fibrillation may be regarded as partly of ventricular and partly of auricular origin, or, as is frequently the case, as due to an interference of the two tractions. It is owing to their dual origin that they are more numerous and distinct when recorded from the same animal without changing the position or pressure of the receiving apparatus.

## EXPLANATION OF PLATES.

## PLATE 5.

FIG. 1. Intra-auricular pressure, jugular pulse, and auricular myogram, showing transition from fine to coarse fibrillation and from this to coordinated contractions. Diastolic waves of venous pulse (a) occur during fine as well as coarse fibrillation. Shortening of the auricle causes downward movement in this and all other myogram curves. Time 0.2 second.

FIG. 2. Auricular myogram (downstroke in systole) and electrocardiogram, Lead II, showing that diastolic waves of the electrocardiogram do not correspond to mechanical changes of the auricle and that waves are more numerous during fine fibrillation.

## PLATE 6.

FIG. 3. Jugular pulse, auricular myogram, and intra-auricular pressure showing the traction effect of the ventricle on the auricle and venous pulse (p. 27).

FIG. 4. The same as Fig. 3 (p. 28).

## PLATE 7.

FIG. 5. Four segments of a record showing the movements of the superior vena cava (upper curve), a right auricular myogram (middle curve), and an electrogram of the right auricle (lower curve). Time 0.05 second.



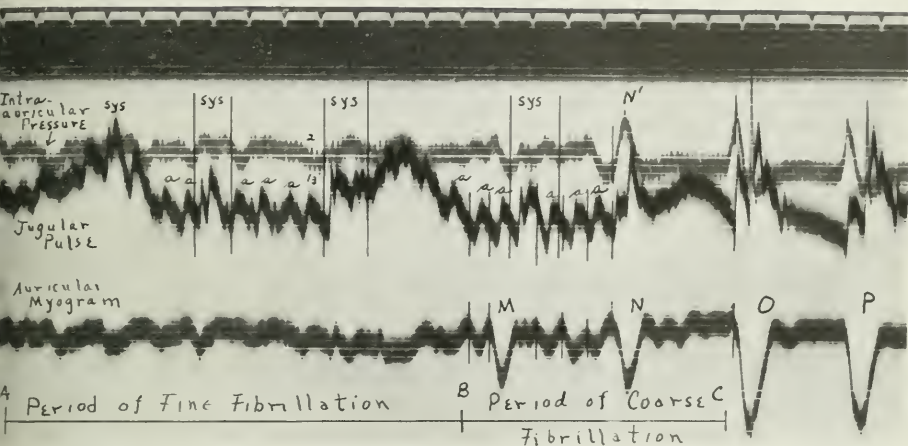


FIG. 1.

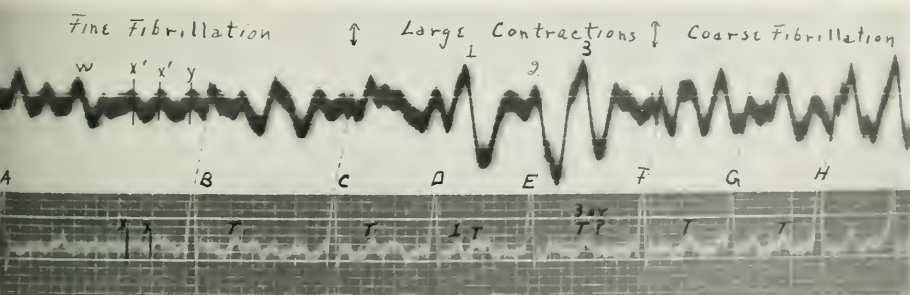


FIG. 2.

(Wiggers and Niles: Diastolic Waves of the Venous Pulse.)





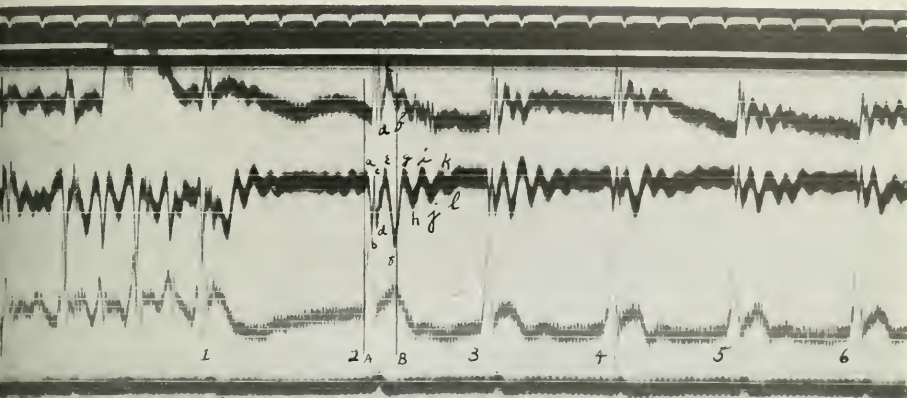


FIG. 3.



FIG. 4.

(Wiggers and Niles: Diastolic Waves of the Venous Pulse.)



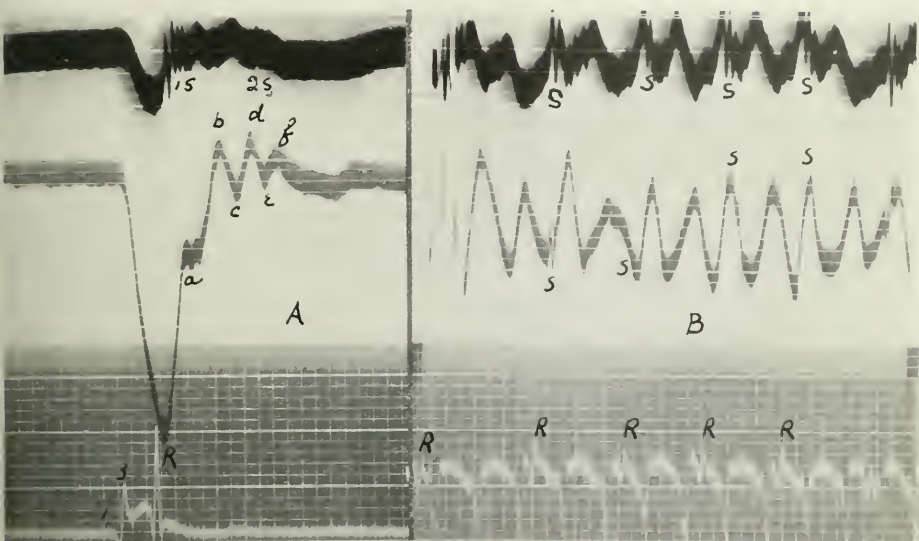


FIG. 5 a.

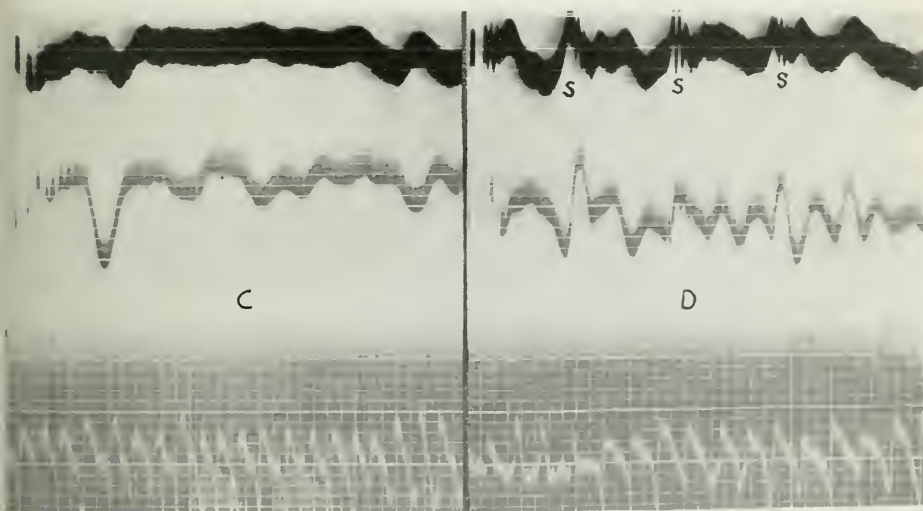


FIG. 5 b.

(Wiggers and Niles: Diastolic Waves of the Venous Pulse.)



# SPIROCHÆTA MORSUS MURIS, N.SP., THE CAUSE OF RAT-BITE FEVER.

## SECOND PAPER.

BY KENZO FUTAKI, M.D., ITSUMA TAKAKI, M.D., TENJI TANIGUCHI, M.D.,  
AND SHIMPACHI OSUMI, M.D.

(From the Imperial Institute for Infectious Diseases, Tokyo, Japan.)

PLATES 8 TO 10.

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We have already reported the finding of a new spirochete,<sup>1</sup> which we believe to be the cause of rat-bite fever and which was present in the blood, skin, and lymph glands of two out of four patients suffering from the disease. Five more cases, in each of which we have been able to find a spirochete, have come under our observation and in two of the patients we found the spirochete in the circulating blood derived from a vein or the punctured skin.

### *Description of the Spirochete.*

*Morphology.*—The spirochete consists of a body and flagella. In stained film preparations of the blood of a patient or inoculated animal, the body is spiral and comparatively short and thick. The size varies from 1.5 to 2  $\mu$  to 5 to 6  $\mu$ ; when the flagella at both ends are included, the length is 6 to 10  $\mu$ . Smaller bodies are found in the blood in greater numbers than large ones, while in lymph nodes slightly larger ones occur. In cultures still larger ones measuring up to 12 to 19  $\mu$ , have sometimes been observed; but minute ones of 1.5 to 2  $\mu$  also appear.

In our previous report we stated that the organism in the patient's lymph nodes was somewhat larger than *Spirochæta pallida*, but

<sup>1</sup> Futaki, K., Takaki, I., Taniguchi, T., and Osumi, S., *J. Exp. Med.*, 1916, xxiii, 249.

smaller than *Spirochæta duttoni* and *obermeieri*. It is thicker also than *Spirochæta pallida* and presents usually one curve per micron, the curves being regular and sharp. The number of curves ranges from one and a half to six; occasionally ten to nineteen curves appear. Other exceptions are as follows: in cultures the angle of the curves is obtuse; some spirochetes show one curve in  $2\ \mu$ . In rare instances, especially in cultures in which degeneration quickly occurs, the curves become irregular. The characteristics are the same whether living preparations or stained films are studied. An undulating membrane has not been found. The protoplasm stains equally well, regardless of the stain used. The ends of the body gradually become pointed and pass into the slender flagella. In most cases flagella project from both ends; sometimes from one only. Rarely two flagella occur at one end. The flagella have approximately the same thickness and measure 2 to  $3\ \mu$ .

*Staining.*—The spirochetes stain readily. Giemsa's stain, Löf-  
fler's methylene blue, Ziehl's solution, and aqueous gentian violet all stain the organisms readily. They are, however, Gram-negative. Silver impregnation succeeds easily. The flagella may or may not become stained with Giemsa's stain or silver solution. India ink, used according to Burri, is very effective in revealing the flagella. With Giemsa's stain the bodies take a deep violet-red coloration. Levaditi's method of silver impregnation is most suitable for demonstrating the presence of the spirochete in the tissues. By this method the bodies appear thick and the curves sharper, giving the organism a spindle-like appearance. In this case, the spirochetes can be distinguished only by means of the flagella which assume a yellowish brown color. In some culture organisms one or more chromatin bodies have been detected by means of Giemsa's stain.

*Locomotion.*—Usually it is difficult to find the spirochetes in the blood or in the lymph of patients even by means of dark-field illumination, as the number is small. Recently, however, we observed in one case out of five, spirochetes in blood from the punctured skin. We have, on the other hand, experienced difficulty in finding the organisms in thick blood preparations made according to Koch's method. In the inoculated mouse it is not infrequent to find from one to five spirochetes in a single field of blood or lymph. Hence the number of



spirochetes is far less than in other spirochetal infections communicated to animals.

The light refraction is weak and the motion is like that of a vibrio. The organism moves rapidly across the field of vision; but the cultivated spirochetes with long bodies are, on the contrary, inactive, making only wriggling movements.

Movements may be clearly discerned in specimens studied according to Shimamine's<sup>2</sup> method. The agar medium, after having been dissolved and cooled to 45°C., is mixed with fresh blood containing spirochetes. This mixture is placed under a cover-glass and the spirochetes can be seen fixed in the agar; capillary preparations are then made. The movements are spasmodic, usually spiral, and freely pass backwards and forwards. Comparatively long organisms undergo flexion and rotation. Meanwhile, whipping, winding, stretching, and darting motions of the flagella can be seen. When the bodies are very small it is often impossible to distinguish the various movements.

The spirochete examined microscopically under a strong light becomes quiescent comparatively soon. In animal blood at a temperature of 22–37°C., it ceases to move in about 24 to 48 hours. In this condition it is not recognizable under the dark-field microscope.

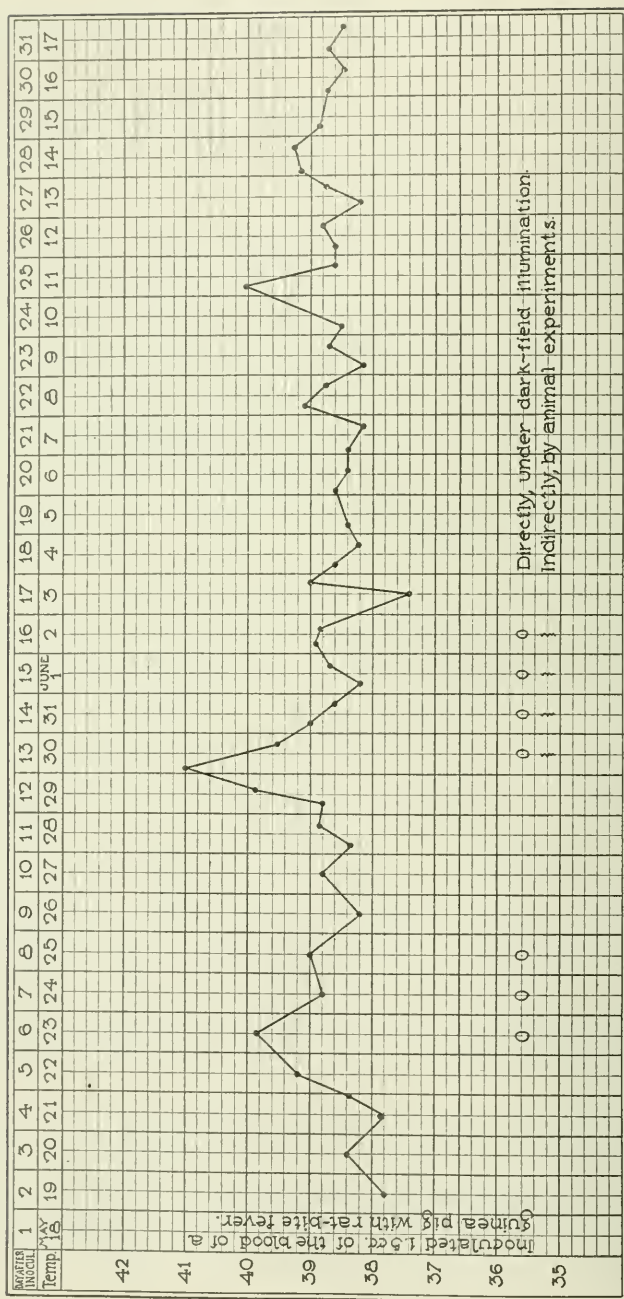
Mice, house rats, white rats, guinea pigs, and monkeys are suitable for the experiments, but mice are the best, especially when human material is to be inoculated directly into animals. White rats are the next best. Guinea pigs and monkeys frequently yield no result.

Spirochetes which have passed through the blood of mice or rats several times are highly virulent for other animals. This virulence depends, of course, largely upon the number of spirochetes inoculated.

After the inoculation of a mouse with human material, 7 days at the earliest, and several weeks or a month at the latest elapse before the organisms can be discovered in its blood. But if a mouse is in-

<sup>2</sup> Shimamine's medium is prepared as follows: 0.5 to 0.75 gm. of sodium nucleate and 100 cc. of horse serum are shaken until the former is completely dissolved, after which carbon dioxide is passed through the solution for 3 or 4 minutes, until the serum becomes transparent. The liquid is heated on 3 successive days, for about an hour at 60°C. On the 4th day it is heated at 65°C. for about 30 minutes, when it separates into a fluid and a coagulated portion.





TEXT-FIG. 1. *Pithecius fuscatus* (Blyth) inoculated with the blood of a guinea pig suffering from rat-bite fever.

oculated from an infected mouse, the spirochetes usually begin to appear in 4 days. Mice usually survive the experiment and retain the spirochetes for several months after the inoculation. Although the other animals generally survive the disease, death occurs now and then from various causes. Swelling of the spleen, hyperemia, congestion and swelling of the liver, or hemorrhage of the kidneys and lungs are often observed. Many of the organisms are also found in the liver or kidneys of animals as well as in their blood. The type of fever produced in the monkey, by the inoculation of spirochetes of rat-bite fever, is not typical of the fever in man. However, the type of fever in the monkey caused by inoculation with the spirochetes of relapsing fever is not typical of relapsing fever in man.

*Propagation of the Spirochetes in the Human Body.*

As cases of rat-bite fever are rare—only eight have come under our observation—and as we have not yet examined a fatal case,<sup>3</sup> our investigation is still far from complete. In the early stages the spirochetes are found in the local eruption of the skin and in the enlarged lymph nodes, whence they seem gradually to invade the blood.

In relapsing fever the organisms are numerous during the febrile, but decrease greatly in number or disappear altogether during the afebrile periods. When animals are inoculated with blood from a patient suffering from rat-bite fever during the afebrile period or when there is light fever, the spirochetes are present. However, it is always difficult to find them as they are few in number.

EXPERIMENTAL.

A. The striking clinical effect of salvarsan upon rat-bite fever has already been reported by Taniguchi and Hata,<sup>4</sup> whose experiments we have confirmed. We divided into three groups a number of mice in the blood of which the spirochetes had been demonstrated. The first group was kept as a control, and each animal in the other two

<sup>3</sup> A postmortem has recently been reported from the Kyushu Imperial University by Drs. Kaneko and Okuda. They claim to have found the same organism as ours in the intestines, and especially in the kidneys.

<sup>4</sup> Hata, S., *Münch. med. Woch.*, 1912, lix, 854.

groups was injected with  $\frac{1}{600}$  to  $\frac{1}{800}$  gm. of salvarsan for every 20 gm. of body weight (Table I).

TABLE I.

*Experiments with Salvarsan.*

*Hypodermic Injection of 0.2 Cc. of Blood Containing Spirochæta morsus muris.*

Mouse No. Body weight.	1	2	3	4	5	6	7	8	9
	Control.			12 gm.	13 gm.	12 gm.	12 gm.	13 gm.	14 gm.
Dose per 20 gm. of body weight.				$\frac{1}{600}$ gm.			$\frac{1}{800}$ gm.		
No. of spirochetes.									
1st day (Mar. 2).	—	+	+	+	+	+	+	+	+
2nd "	+	+	+	—	—	—	—	—	—
3rd "	+	+	+	—	—	—	—	—	—
4th "	+	+	+	—	—	—	—	—	—
5th "	+	+	+	—	—	—	—	—	—
6th "	+	+	+	—	—	—	—	—	—
7th "	+	+	+	—	—	—	—	—	—
8th "	+	+	+	—	—	—	—	—	—
9th "	+	+	+	—	—	—	—	—	—
10th "	+	+	+	—	—	—	—	—	—
11th "	+	+	+	—	—	—	—	—	—
12th "	+	+	+	—	—	—	—	—	—
13th "	+	+	+	—	—	—	—	—	—
14th "	+	+	+	++	—	—	—	—	+
15th "	+	+	+	+	—	—	—	+	+
16th "	+	+	+	+	+	—	—	+	+

On the following day, though the spirochetes were found as before in the blood of the control animals, none were discovered in the other groups. This result continued until the 14th day, when about one-third of the animals showed a relapse; by the 17th day almost two-thirds had relapsed, and after 2 months 83 per cent showed spirochetes. A similar phenomenon has been witnessed in human beings.

*Occurrence of the Spirochete in Nature.*

We examined the blood of forty-three rats under dark-field illumination, and in one only, namely, a *Mus rattus alexandrinus* (roof rat) did we detect spirochetes. With the blood of this rat a white mouse was inoculated successfully. In respect to morphology, staining,

locomotion, and infectivity the spirochetes present in the roof rat are identical with those obtained from human cases of rat-bite fever. Yet we have not discovered the organism in the mouths of healthy house rats or in the saliva of house rats and other animals in whose blood the spirochete exists.<sup>5</sup> The spirochete found in a *Mus decumanus* by Carter<sup>6</sup> in 1887 and again by Mezinescu<sup>7</sup> in 1909 may be the same as ours. Moreover, Borrel,<sup>8</sup> Calkins,<sup>9</sup> and others have described spirochetes in cancerous and healthy mice. In one instance the description fitted the organism we are considering, but it has not been detected up to the present in healthy mice or guinea pigs in Japan.

B. We discovered our organism in November, 1915. Soon after, Ishiwara and his associates succeeded in cultivating outside the body a spirochete which was transmitted by the bite of a house rat to a guinea pig. In this case the spirochete-containing blood of the guinea pig was used. We have made similar observations on a somewhat larger scale. Some fifteen house rats having bitten guinea pigs, we examined the blood of the latter and found in one instance spirochetes which were identical with those present in rat-bite fever in man.

C. From the fact that patients suffering from rat-bite fever have invariably been bitten by rats and also that our spirochetes are found both in house rats and in human patients with rat-bite fever, it may be inferred that the spirochete is transmitted from these animals to the human body, and the experiments described above confirm this view. But as the organisms have not been detected in the saliva but only in the blood of rats, the source of the infectious organism probably is from the blood which, owing to hemorrhage from the gums, enters the saliva of the rat.

D. Various methods have been tested for the cultivation of the

<sup>5</sup> We owe to Drs. Ishiwara, Otawara, and Tamura valuable specimens of spirochetes from two rats—a *Mus decumanus* and a *Mus rattus alexandrinus*—in which they detected the organism.

<sup>6</sup> Carter, V., *Sc. Mem., Med. Officers Army of India*, 1887, iii, 45.

<sup>7</sup> Mezinescu, D., *Compt. rend. Soc. biol.*, 1909, lxvi, 58.

<sup>8</sup> Borrel, A., *Compt. rend. Soc. biol.*, 1905, lviii, 770.

<sup>9</sup> Calkins, G. N., and Clowes, G., *J. Infect. Dis.*, 1905, ii, 555.

spirochete, including especially Noguchi's and Shimamine's<sup>2</sup> employed for the cultivation of *Spirochæta pallida*. We have succeeded in cultivating our organism with the latter method, the first appearance of growth being noted after 14 days at 37°C. The inoculation is made by thrusting into the medium, thus prepared, a capillary tube impregnated with the blood of an animal containing the spirochetes. The tubes are not paraffined, and are left in the thermostat at 37°C. for 2 weeks. The growth is attended neither by liquefaction nor other obvious changes in the medium and no odor is developed. Multiplication is detected first by the dark-field microscope, and secondly by stained preparations. The pathogenicity of the cultures is under investigation.

The cultures show the following characteristics. The maximum number of spirochetes found in a microscopic field of the blood used for inoculation was three to four; of the culture, six to ten. The blood having been placed in the center of the medium, the spirochetes are diffused in the medium to within 1 cm. of the surface. Should the spirochetes not multiply in the culture those inoculated disappear in a week at the longest. Thus far a second culture generation has not been secured.

The size of the culture organisms varies greatly—the short ones measure from 1.5 to 2  $\mu$ , the large ones from 12 to 19  $\mu$ . The spirals are fewer in number (one curve in 2  $\mu$ ) than in the blood spirochetes. The total number of curves varies from 1 to 1.5 to 19. The larger individuals in cultures have not been found either in the blood or lymph glands.

The culture spirochetes possess the same staining reaction as those of the blood. All the aniline dyes stain them, and Giemsa's solution gives them a deep violet-red tint. The flagella also stain distinctly. The larger organisms seem to divide transversely; vertical division has not been observed. Between the bodies slender, light colored mid-lines have been seen. In one instance one body was noted to give rise to four bodies. We have occasionally seen organisms with irregular spirals unevenly stained, which might be considered as degenerate forms. Once we observed from one to several deep red refractive chromatin corpuscles scattered among the culture spirals. In one instance only have we seen two flagella at one end of the body.



The culture organisms are motile—movements of the short individuals are brisk and similar to those of vibrios; the larger spirochetes exhibit slow, wriggling or whipping movements. 0.1 per cent sodium taurocholate and saponin dissolves the organism.

As regards classification, we believe that the spirochete belongs to the protozoa and not to the bacteria. We have not yet succeeded in obtaining a second generation of the culture.

Spirochetes have been described as occurring in rats or mice either in the blood or in tumors by many investigators (Carter,<sup>6</sup> Borrel,<sup>8</sup> Calkins<sup>9</sup> Wenyon,<sup>10</sup> Breinl and Kinghorn,<sup>11</sup> MacNeal,<sup>12</sup> Tyzzer,<sup>13</sup> Gaylord,<sup>14</sup> Deetjen,<sup>15</sup> Löwenthal,<sup>16</sup> Mezinescu,<sup>7</sup> Negre, (1910), and Hoffmann (1906). Although only a part of the descriptions suffices for certain identification, we have not been able to identify our organism with any of them. Moreover, in the case of none of the spirochetes described has the question of human infection entered. Hence we have concluded to offer as a designation of the spirochete of rat-bite fever the name *Spirocheta morsus muris*.

Ogata believes that rat-bite fever is caused by an aspergillus; Shikami<sup>17</sup> that it is caused by a telosporidia; Middleton,<sup>18</sup> by a diplococcus; Proescher,<sup>19</sup> by bacteria; and Schottmüller,<sup>20</sup> by a streptothrix. The streptothrix theory of Schottmüller was supported separately by Blake<sup>21</sup> and Tileston, and also by Tunncliffe.

By rat-bite fever we mean a disease having the symptoms described above, but the fever does not necessarily include all diseases related to rat bites. Hence we consider that the cause of rat-bite fever in this sense is the spirochete that we have described. In Schottmüller's two cases in which the victims fell ill without an incubation period, the disease may have been caused by a streptothrix, but it is not what we should call rat-bite fever. Tileston's cases were appar-

<sup>10</sup> Wenyon, C. M., *J. Hyg.*, 1906, vi, 580.

<sup>11</sup> Breinl, A., and Kinghorn, A., *Lancet*, 1906, ii, 651.

<sup>12</sup> MacNeal, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1906-07, iv, 125.

<sup>13</sup> Tyzzer, E. E., *Proc. Soc. Exp. Biol. and Med.*, 1906-07, iv, 85.

<sup>14</sup> Gaylord, *Ann. Rep. Cancer. Lab., New York State Dept. Health*, 1907, viii, 34.

<sup>15</sup> Deetjen, H., *Münch. med. Woch.*, 1908, lv, 1167.

<sup>16</sup> Löwenthal, W., *Berl. klin. Woch.*, 1906, xliii, 283.

<sup>17</sup> Shikami, *Z. med. Ges. Tokyo*, 1909.

<sup>18</sup> Middleton, G. S., *Lancet*, 1910, i, 1618.

<sup>19</sup> Proescher, F., *Berl. klin. Woch.*, 1912, xlix, 841.

<sup>20</sup> Schottmüller, H., *Derm. Woch.*, 1914, lviii, Suppl. 77.

<sup>21</sup> Blake, F. G., *J. Exp. Med.*, 1916, xxiii, 39.

ently the same as ours, but he failed to detect the spirochete. Blake's case differs from our cases of rat-bite fever in that the lymph glands were not swollen, the rash was temporary, and the fever was not paroxysmal; it may properly be regarded as a case of streptothrix sepsis caused by the bite of a rat.

#### SUMMARY.

1. Since our first report on the discovery of the cause of rat-bite fever, we have been able to prove the existence of the same spirochete in five out of six more cases which have come under our observation.

2. The clinical symptoms of rat-bite fever are inflammation of the bitten parts, paroxysms of fever of the relapsing type, swelling of the lymph glands, and eruption of the skin, all occurring after an incubation period usually of from 10 to 22 days, or longer.

3. Our spirochete is present in the swollen local lesion of the skin and the enlarged lymph glands. But as the spirochetes are so few in number it is exceedingly difficult to discover them directly in material taken from patients. It is therefore better to inoculate the material into a mouse. In some cases the organism is found in the blood of the inoculated animal after a lapse of 5 to 14 days, or at the latest 4 weeks.

4. Generally speaking, the spirochetes present thick and short forms of about 2 to 5  $\mu$  and have flagella at both ends. Including the flagella, they measure 6 to 10  $\mu$  in length. Some forms in the cultures reach 12 to 19  $\mu$  excluding the flagella. The curves are regular, and the majority have one curve in 1  $\mu$ . Smaller ones are found in the blood and larger ones in the tissues.

5. The spirochetes stain easily. With Giemsa's stain they take a deep violet-red; they also stain with ordinary aniline dyes. The flagella, too, take Giemsa's stain.

6. The movements of our spirochetes are very rapid, resembling those of a vibrio, and distinguish them from all other kinds of spirochetes. When, however, the movements become a little sluggish, they begin to present movements characteristic of ordinary spirochetes.

7. For experimental purposes, mice, house rats, white rats, and monkeys are the most suitable animals. Monkeys have intermittent fever after infection, and spirochetes can be found in their blood, but



they are not so numerous as in the blood of mice. Mice are the most suitable animals for these experiments, and they appear, as a rule, to escape fatal consequences.

8. The spirochete is markedly affected by salvarsan.

9. The organism is not present in the blood of all rats, and there is no relation between the species of the rat and the ratio of infection. We have never found the spirochete in healthy guinea pigs or mice. By permitting a rat infected with the spirochete to bite a guinea pig, the latter develops the disease.

10. We have succeeded in cultivating the spirochete in Shimamine's medium.

11. Among the spirochetes described in the literature or discovered in the blood of rats and mice, there may be some resembling our spirochete, but none of the descriptions agree with it fully. Hence we have named our organism *Spirochæta morsus muris* and regard it as belonging to the Spironemacea (Gross) of the nature of treponema.

12. The spirochete can be detected in the bodies of patients. In seven cases out of eight, it disappears on recovery, only to reappear during the relapse.

13. The spirochete can be detected in about 3 per cent of house rats. These facts enable us to identify the cause of the disease.

14. There may be other causes than the spirochete for diseases following the bite of a rat. The cause, however, of rat-bite fever in the form most common in Japan is, we believe, the spirochete which we have described.

In conclusion we wish to express our indebtedness to Dr. Aoyama, former Director of the Imperial Institute for Infectious Diseases, and Dr. Hayashi, the present Director; to Dr. Miura who named the spirochete; to Professor Nagayo, Assistant Professor Ishiwara, and Drs. Manabe, Miyagawa, Mitamura, Matsuyama, and Katayama, for valuable suggestions; and to Dr. Oba, and Messrs. Itakura and Isosaki for the help they have given us.

#### EXPLANATION OF PLATES.

##### PLATE 8.

FIG. 1. Section of a lymph gland from a patient with rat-bite fever. Impregnated with silver nitrate according to Levaditi's method.  $\times 1,250$ .

FIG. 2. Section of the lung of a mouse inoculated with venous blood from a patient with rat-bite fever. The length of the body of the spirochete is  $2.2\ \mu$ ; including the flagella it is  $6\ \mu$ . Silver impregnation.  $\times 1,500$ .

FIG. 3. A spirochete from a guinea pig with experimental rat-bite fever. The length of the body is  $4\ \mu$ .; with the flagella,  $8.5$ . Giemsa's stain.  $\times 1,500$ .

FIG. 4. Spirochetes from a guinea pig with experimental rat-bite fever. The length of the bodies varies from  $2.2$  to  $4\ \mu$ . Giemsa's stain.  $\times 1,250$ .

#### PLATE 9.

FIG. 5. *Spirochæta morsus muris* from a mouse inoculated with the blood of a patient with rat-bite fever. The length of the body is  $4\ \mu$ ; with the flagella it measures  $9\ \mu$ .  $\times 3,600$ .

#### PLATE 10.

FIG. 6. First column, *Spirochæta morsus muris* from the blood and tissues of human patients and inoculated animals. Second and third column, cultures of *Spirochæta morsus muris*.

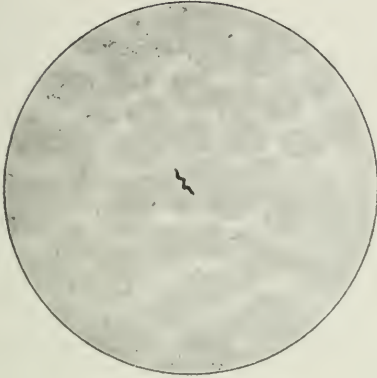


FIG. 1.

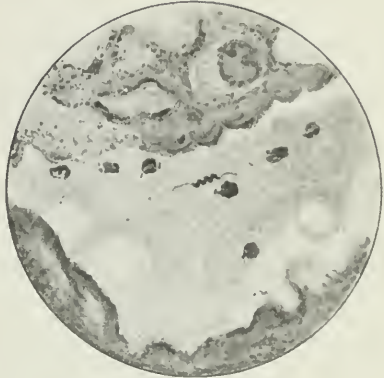


FIG. 2.

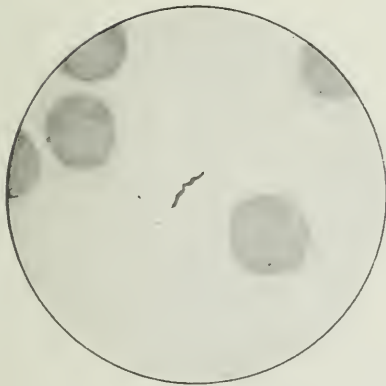


FIG. 3.

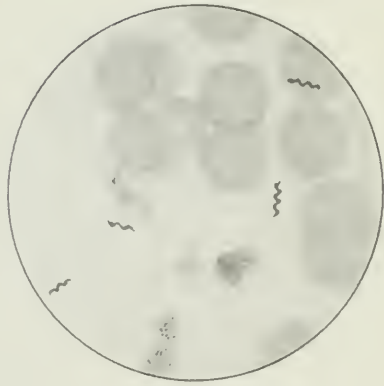


FIG. 4.

(Futaki, Takaki, Taniguchi, and Osumi: Cause of Rat-Bite Fever.)





FIG. 5.

(Futaki, Takaki, Taniguchi, and Osumi: Cause of Rat Bite Fever.)



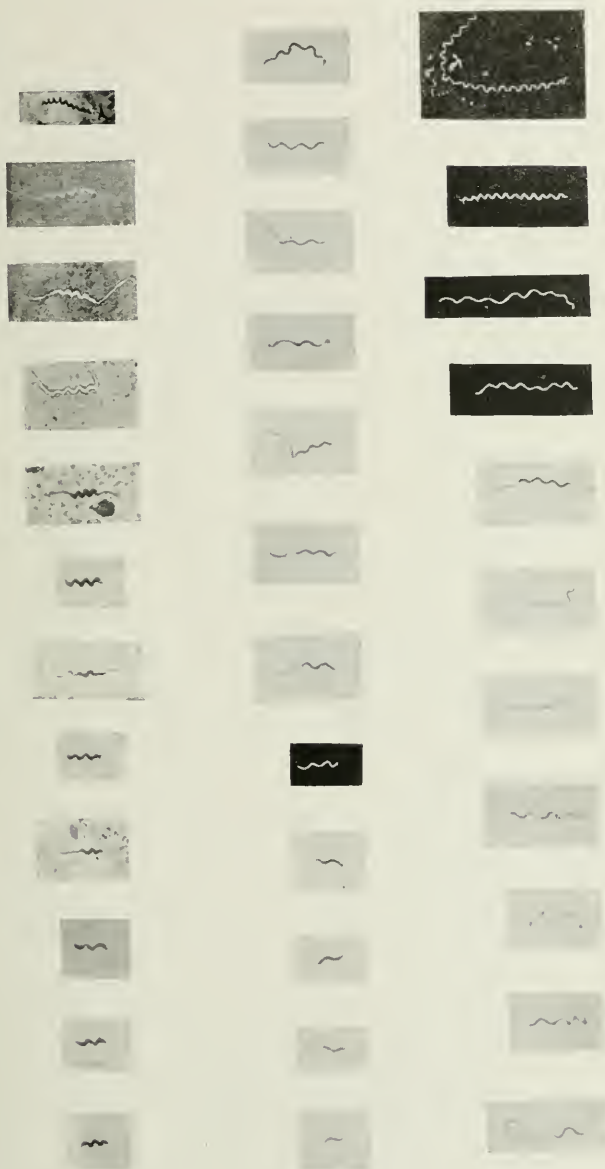


FIG. 6.

(Futaki, Takaki, Taniguchi, and Osumi: Cause of Rat-Bite Fever.)





# EXPERIMENTAL RAT-BITE FEVER.

## FIRST REPORT.\*

By KIKUTARO ISHIWARA, M.D., TOYOITSIRO OHTAWARA, M.D., AND  
KOTARO TAMURA.

(From the Imperial Institute for Infectious Diseases, Tokyo, Japan.)

### PLATE 11.

(Received for publication, August 7, 1916.)

### INTRODUCTION.

At least eighty-one cases of human rat-bite fever have, according to Blake,<sup>1</sup> been reported by European, British, American, and Japanese investigators. Only two of the reports, however, those of Miura and Toriyama<sup>2</sup> and of Blake, include the pathological anatomy of the disease. Ogata<sup>3</sup> attributes rat-bite fever to an aspergillus, Shikami<sup>4</sup> attributes it to a telosporidia, Middleton<sup>5</sup> to a diplococcus, Proescher<sup>6</sup> to a bacillus, and Schottmüller<sup>7</sup> and Blake to a streptothrix.

Ogata inoculated two guinea pigs with a freshly excised piece of a swollen lymph gland taken from a patient with rat-bite fever; both animals died about 3 weeks later. The swollen glands of the animals were inoculated into other guinea pigs which also died about 3 weeks after the inoculation. In 1909 Ogata caused a *Mus decumanus* to bite two guinea pigs in the leg; the guinea pigs developed fever and swelling and congestion of the bitten parts and finally succumbed. Autopsy of all these animals showed swelling and congestion of the lymph glands and swelling of the kidneys to be the chief lesions, although pneumonia was present in some instances. From these experiments Ogata concluded that rat-bite fever could be produced experimentally in animals by the bite of rats.

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\* This paper was presented at the meeting of the Japanese Hygienic and Bacteriological Society on March 3, 1916.

<sup>1</sup> Blake, F. G., *J. Exp. Med.*, 1916, xxiii, 39.

<sup>2</sup> Miura, M., and Toriyama, N., *Z. med. Ges. Tokyo*, 1897, xi, 1059.

<sup>3</sup> Ogata, *Mitt. med. Fakult. Univ. Tokyo*, 1911, ix, 1913; 1913-14, xi, 179.

<sup>4</sup> Shikami, *Z. med. Ges. Tokyo*, 1909.

<sup>5</sup> Middleton, G. S., *Lancet*, 1910, i, 1618.

<sup>6</sup> Proescher, F., *Berl. klin. Woch.*, 1912, xlix, 841.

<sup>7</sup> Schottmüller, H., *Derm. Woch.*, 1914, lviii, Suppl. 77.

One of us had the opportunity of assisting at these experiments, and noted pathological changes in the adrenals of the animals, but Ogata made no mention of them in his report.

In March, 1915, one of us undertook the serological study of guinea pigs bitten by rats. In November of the same year Futaki and his collaborators examined under dark-field illumination and by Burri's method the tissue fluid of excised skin and that of punctured lymph glands of two patients suffering from rat-bite fever. They also examined the excised lymph gland of one of the patients, stained by Levaditi's method, and found that it contained a number of spirochetes. About the same time we examined the excised viscera of guinea pigs which we had kept for study from the spring of that year and found a spirochete in sections of the adrenals of several animals, stained according to Levaditi's method. Futaki<sup>8</sup> and his coworkers announced the results of their investigation at a meeting of the Tokyo Medical Society on November 20, 1915, and we reported our findings at the same meeting. We promptly found the same spirochetes in living animals which were under observation and described them in a second report made before the Tokyo Medical Society on February 5, 1916.

Ogata was the first to transmit rat-bite fever to guinea pigs by causing rats to bite them, and we have confirmed his experiments.

#### EXPERIMENTAL.

We caught many rats by means of wire nets and kept them under observation in the laboratory. The rats were divisible into two classes, those that would and those that would not bite when irritated from time to time by means of a small metal rod. Rats which did not bite in one test would, as a rule, not bite in another. Moreover, only a part of the rats were capable of conferring the rat-bite infection, because a rat which did not confer the disease to one animal by biting did not confer it to another; conversely a rat which conveyed the disease by biting one animal would also convey it by biting another. This led us to conclude that only certain rats tend to bite and of those only a part convey the infection of rat-bite fever.

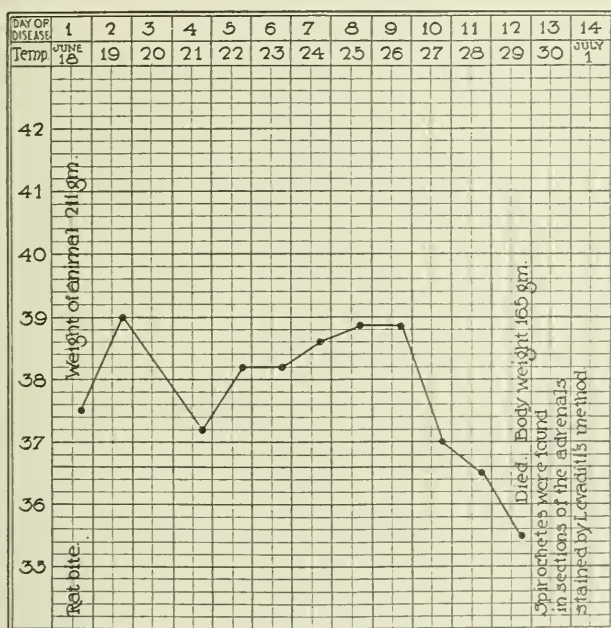
The test animals were guinea pigs the legs of which were exposed. About 80 rats were employed, one-half of which were made to bite, and of this number only about ten individuals, all *Mus decumanus*, caused experimental rat-bite fever. Thus far we have not discovered the exact conditions under which the disease is transmitted, and we have not detected the specific spirochete in the mouths

<sup>8</sup> Futaki, Takaki, Taniguchi, and Osumi, *Z. med. Ges. Tokyo*, 1915.

of rats. In one rat, however, we detected a few spirochetes of the same form in the blood.

### *Clinical Course.*

The symptoms and course of the disease in the guinea pig were as follows: The bitten parts became swollen within 2 or 3 days, and in severe cases the affected legs reached three times the natural size. The bite healed promptly, but the swelling continued, sometimes de-

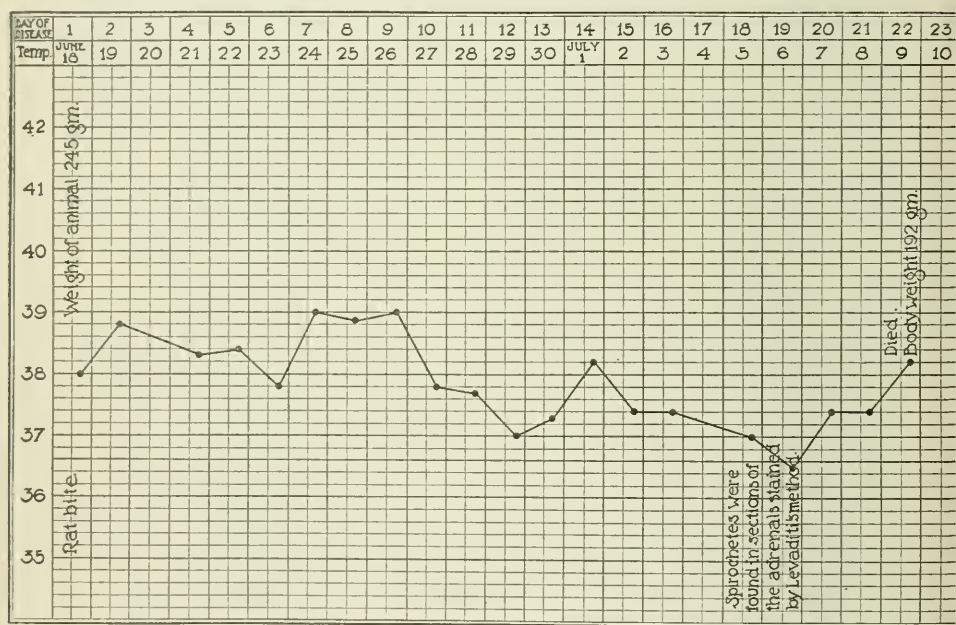


TEXT-FIG. 1. Temperature chart of Guinea Pig 1 (original generation).

creasing, until the death of the animal. The skin of the leg became cyanosed and the subcutaneous lymph glands became palpable. Within 24 hours after the bite a rise in temperature occurred, but this was soon followed by a fall to normal. A second rise of temperature took place 6 to 7 to 10 days later, only to fall again soon. This rise and fall might be repeated, but in this respect the experimental condition does not run so regular a course as human rat-bite fever.

Weight was gradually lost, but an erythema did not appear. The guinea pigs which showed this condition invariably died. The course of the fever was not uniform, but tended to extend over 3 weeks. The longest course was something over 5 weeks, the shortest less than 2 weeks. Text-figs. 1, 2, and 3 show the progress of events in three instances.

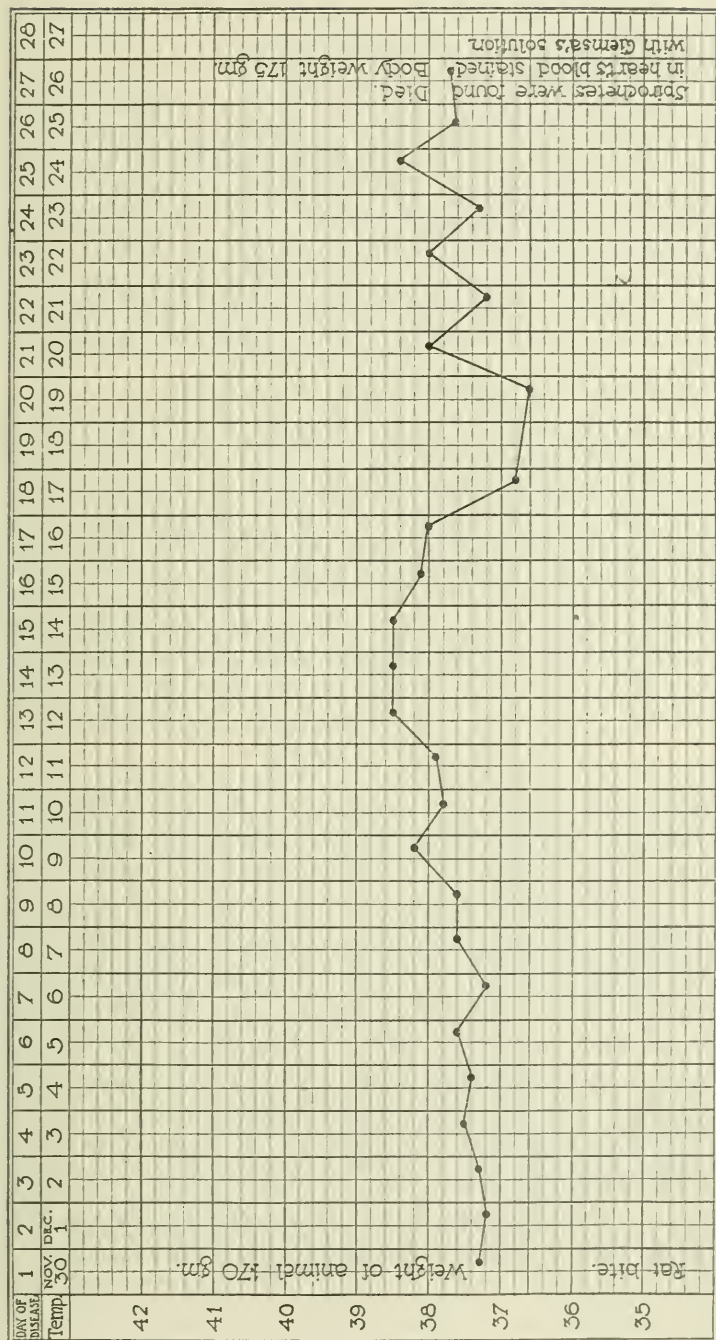
Guinea Pig 1 (Text-fig. 1) was bitten by *Mus decumanus* 4 on June 18, 1915. On the 2nd day the bitten parts became swollen and on the 4th day the enlarged inguinal glands on the affected side were palpable.



TEXT-FIG. 2. Temperature chart of Guinea Pig. 2 (original generation).

The anatomical appearances are described below. Two fresh guinea pigs were inoculated with emulsions made from the lymph glands and two with emulsions made from brain tissues of this animal. Spirochetes were found in sections of the adrenals stained according to Levaditi's method. Aerobic cultures made after the animal's death from the intraperitoneal exudate, heart's blood, fluids of brain tissues and excised tissues taken from the section through the bitten parts were negative, as were also the stained cover-glass preparations.





TEXT-FIG. 3. Temperature chart of Guinea Pig 3 (original generation).



Before inoculation we always examine the animals' blood microscopically, after treating it with acetic acid.

Guinea Pig 2 (Text-fig. 2) was bitten by the same rat on the same day as No. 1. On the 2nd day the bitten parts became swollen; the swelling decreased soon after, but it increased again on the 9th day. Enlargement of the inguinal glands could be detected on palpation. On the 14th day the swelling of the bitten parts again decreased. The anatomical appearances were identical to those described below. Aerobic cultures made from the excised tissues of the bitten parts, the intraperitoneal fluid, heart's blood, and brain tissue fluid were negative. Stained cover-glass preparations were also negative. Two fresh guinea pigs were infected with emulsions made from lymph glands and brain tissues. Excised tissues of the adrenals stained according to Levaditi's method were positive for spirochetes.

Guinea Pig 3 (Text-fig. 3) was bitten on November 30, 1915, by another *Mus decumanus*. The next day the leg was swollen to three times the normal size and developed a dark cyanotic color. From the 10th day on the fever progressed as shown in Text-fig. 3. The animal died on the 28th day after being bitten.

*Autopsy.*—Weight of animal not decreased. The leg remained swollen. Swelling and congestion of the subcutaneous and internal lymph nodes were also present. Both adrenals were markedly swollen and congested. The kidneys of both sides showed congestion of the cortex. Aerobic cultures from the peritoneal fluid and heart's blood were sterile. Further inoculation into guinea pigs with the heart's blood and emulsion of the mesenteric lymph nodes gave positive results. Spirochetes were found in the heart's blood stained by Giemsa's solution.

### *Anatomical Changes.*

The subcutaneous lymph glands and the inguinal and axillary glands were swollen and congested. The periglandular tissues and the mesenteric and retroperitoneal glands were also congested. The liver and lungs were congested, the latter often showing pneumonic foci. The spleen showed no apparent macroscopic change. The kidneys were more or less swollen, hemorrhage being often noted under the capsule. The sectioned surface showed edema, and small hemorrhagic spots were observed in the cortical parts. The adrenals were congested and showed small hemorrhagic spots under the capsules. In the peritoneal cavity a hemorrhagic exudate was sometimes found. The bladder and gall-bladder were usually full. The pia mater was congested. The important points to be noted among the postmortem appearances are: swelling of the bitten parts, swelling and congestion of the lymph gland system, and congestion and hemorrhage of the

adrenals and kidneys. The changes in the adrenals were not remarkable in cases where the infected guinea pigs died after a long illness.

Microscopic sections of the kidneys stained with hematoxylin and eosin showed the capillary vessels, especially those of the cortex, to be enlarged and congested, with occasional hemorrhagic spots. The convoluted uriniferous tubules and glomeruli showed a degree of hyaline degeneration of the cells, as in acute nephritis. In the adrenals the capillary vessels were enlarged and congested, and small hemorrhagic spots were observed. The capillary vessels in the lungs and liver were also distended and congested.

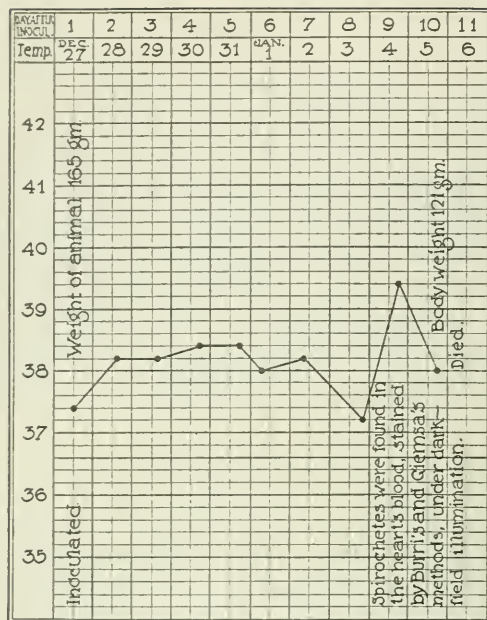
### *Further Inoculation Experiments.*

When a guinea pig died of the fever, emulsions were made from the lymph and adrenal glands, brain substance, heart's blood, etc. The inoculation of these emulsions into the subcutaneous tissues or peritoneal cavities of fresh guinea pigs, always caused the death of the animals after giving rise to such symptoms as high temperature, swelling of the lymph glands, and a decrease in body weight. In these animals, which we called the first generation, the disease ran a shorter course than in the original generation. Some of the experiments are given below.

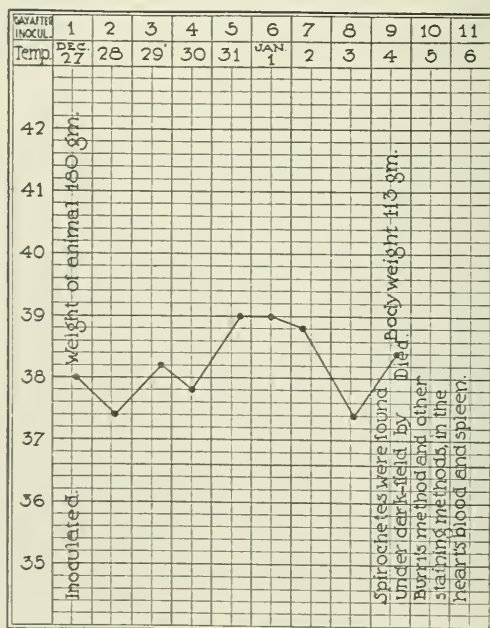
Guinea Pig 4, first generation, (Text-fig. 4) was inoculated on December 27, 1915, by injecting the heart's blood taken from Guinea Pig 3, original generation, intraperitoneally. At autopsy swelling and congestion of the inguinal, axillary, mesenteric, and retroperitoneal glands were found. No changes were observed in the peritoneal cavity where the inoculation had been made, but the changes in the kidneys and adrenals were typical. Spirochetes were found in the preparations made from the heart's blood stained with Burri's and Giemsa's solutions and also examined under dark-field illumination. Four fresh guinea pigs were inoculated with the heart's blood of this animal.

Guinea Pig 5, first generation, (Text-fig. 5) was inoculated subcutaneously with an emulsion made from the mesenteric lymph node of Guinea Pig 3, original generation, at the same time as Guinea Pig 4.

*Autopsy.*—The inguinal, axillary, and mesenteric lymph nodes were swollen and congested. The pathological changes of the kidneys and adrenals were typical. Spirochetes were found under dark-field illumination, by Burri's method and other staining methods, in the heart's blood and spleen.



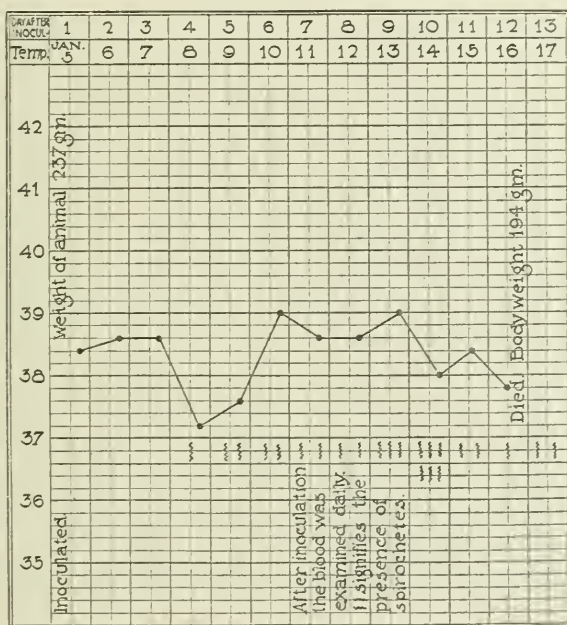
TEXT-FIG. 4. Temperature chart of Guinea Pig 4 (first generation).



TEXT-FIG. 5. Temperature chart of Guinea Pig 5 (first generation).

Further inoculation into mice, white rats, and guinea pigs gave positive results. Spirochetes were found in the heart's blood and films of the spleen under dark-field illumination, with Burri's and Giemsa's stains.

Guinea Pig 6, second generation, (Text-fig. 6) was one of the guinea pigs inoculated into the peritoneal cavity with the heart's blood of Guinea Pig 4 of the first generation. After inoculation we drew blood daily from the ear-lobes, examined it under dark-field illumination, and made stained preparations with Burri's and Giemsa's methods, and other stains. Spirochetes were present in all. The autopsy showed typical pathological changes.



TEXT-FIG. 6. Temperature chart of Guinea Pig 6 (second generation).

At autopsy the guinea pigs of the first generation showed swelling and congestion of the lymph glands and marked changes in the kidneys and the adrenals, as in the original generation.

The inoculation of fresh guinea pigs with the peripheral blood, heart's blood, and emulsion made from lymph glands taken from the guinea pigs of the first generation gave rise to the same symptoms

as in the first generation, and caused the death of the animals. The course of the disease and the result of the postmortem examination were similar to those of the first generation. We obtained the same result from the experiments with material from the animals of the 2nd, 3rd, 4th, and further generations, which indicates that there was no change in the pathogenicity of the spirochetes throughout the generations, which were many in number. It is thus seen that the guinea pig is highly susceptible to the disease.

#### *Experiments on White Rats.*

We inoculated a number of white rats subcutaneously or intraperitoneally with blood taken from an infected guinea pig. The inoculated animals showed no symptoms, but the spirochetes were found to multiply in their blood, as described below. The white rats did not die as a result of the inoculation, but spirochetes always developed in the blood of rats of the new generation. The inoculation into fresh guinea pigs of blood taken from the white rats of the new generation always gave rise to the usual symptoms, causing the death of the animals. In every instance spirochetes were found in the blood. Spirochetes were also found in the blood of mice inoculated with blood from the white rats.

We inoculated white rats with blood containing spirochetes from an infected guinea pig. After spirochetes were observed in the blood of the rats, the legs of two fresh guinea pigs were scratched with the rats' teeth until they bled slightly. One of the guinea pigs died several days later, but no spirochetes were found in the blood. The other guinea pig had fever on the 6th, 11th, 20th, and 25th days, and died on the 27th day.

At autopsy the usual findings were present, though in less degree, and many spirochetes were observed in the heart's blood. We therefore concluded that the cause of infection existed in the mouths of experimentally infected white rats.

#### *Experiments on White Mice.*

Spirochetes were also found in the blood of white mice inoculated with blood from an infected guinea pig. Only a few of the mice died









and no noteworthy macroscopic changes were found at autopsy. Guinea pigs inoculated with the blood of an infected mouse developed the usual symptoms and died. Spirochetes were always found in the blood. White rats inoculated with the blood of the mice also became infected. Many of the spirochetes in the blood of the mice had a large number of spirals.

#### *Experiments on Rabbits.*

We inoculated two rabbits, and no spirochetes were detected in their blood. The number of animals used, however, was too small to allow us to arrive at a definite conclusion.

#### *Experiments on Monkeys.*

We inoculated one *Pithecus irus* (F. Cuvier) and one *Pithecus rhesus* (Audebert) with blood from a guinea pig with experimental rat-bite fever (No. 7). The first monkey developed no symptoms of the disease, but spirochetes were found in the blood of mice and rats inoculated with the monkey's blood. In the *rhesus* monkey intraperitoneal inoculation caused a high temperature on the 5th day (the incubation period), and the lymph glands became swollen and were palpable. The swelling of one or two of the inguinal lymph nodes was especially marked. Erythema was also present. On the 8th day after the inoculation round red patches from 1 to 2 mm. in diameter appeared on the loins and the lower parts of the buttocks. These patches gradually increased in number, 40 to 50 being present on the 12th day. Most of them appeared close together on the skin, and finally coalesced into one another. The surface of the patches was slightly swollen, the red color fading on pressure. There were also some patches on the abdomen. The temperature rose every 2 or 3 days, and recurred six times in 25 days. The fever curve in the *rhesus* resembled that of human rat-bite fever (Text-fig. 7). No spirochetes were found in the films made almost every day from the peripheral blood or in the films from swollen lymph glands and excised lymph node tissue. Spirochetes were present, however, in rats and mice inoculated with the blood and emulsions made from excised glands.

*Cultivation Experiments.*

When an infected guinea pig of the original generation died, we made ordinary aerobic cultures of tissues taken from the site of the bite, the heart's blood, lymph glands, and fluids from the viscera and peritoneal cavity. In these cultures we sometimes found bacilli resembling *Bacillus coli* in form, movement, manner of staining, and pathogenicity. In our opinion, these contaminating bacilli in the cultures came from the animals. No such growth was noticed in other cases. We have not yet succeeded in making cultures from blood containing spirochetes.

*Description of the Organism.*

*Localization of the Spirochetes.*—The first spirochetes that we found were in Guinea Pig 1, bitten by a rat on June 18, 1915. The symptoms, course of the disease, and the postmortem examination indicated that the infection produced in the animal was a typical example of experimental rat-bite fever. Spirochetes were detected in the cortical capillaries and the parenchyma near the capsule in preparations made from excised tissue of the adrenals and stained by Levaditi's method. Spirochetes were observed later in the preserved adrenals of other guinea pigs. In the living animals they were demonstrated in the peripheral blood under dark-field illumination with Burri's and Giemsa's stains and with aniline dyes. At autopsy they were also found in cover-glass preparations of the heart's blood and spleen tissues. The spirochetes were present in all generations, but they were not so numerous as the recurrent spirochetes in the blood of infected mice. The largest number of spirochetes observed in blood films was five or six in one field (Zeiss, oc. 4, oil immersion  $\frac{1}{2}$ ).

*Time of the Appearance of the Spirochetes.*—The symptoms were irregular in the original generation of guinea pigs, as stated above. Spirochetes were found in the peripheral blood only at the last stage of the disease, and before that none could be observed with any degree of accuracy. The animals of the first and later generations, however, displayed the usual symptoms, and spirochetes were observed in the peripheral blood 4 days, rarely 2 days, after inoculation. They gradually multiplied in the blood of guinea pigs, mice, and

white rats, reaching the highest numbers in 8, 10, or 12 days. In white rats and white mice, the spirochetes could be detected more than 2 months after the date of inoculation. Guinea pigs always died within 2 weeks after inoculation.

*Movement.*—In preparations made from the peripheral blood of experimental animals and examined under dark-field illumination, the spirochetes were seen to be transparent, and to possess an active progressive movement. As they passed swiftly out of the field, accurate observation of their morphological characteristics was impossible. They became sluggish after some time, and made spiral, rotating, or other movements. At this stage, the spirochete resembled a woodlouse in shape and appeared to consist of several joints of transverse lines. It was almost impossible to recognize the original shape of a spirochete, and its spiral form may be observed only at a much later period. The spirochetes were still moving actively in the blood 24 hours after the death of the animal.

*Form.*—Preparations made from the heart's blood of guinea pigs and stained according to Giemsa's method showed that the size of the spirochetes varied from 1.6 to 3  $\mu$  in length, 2  $\mu$  being the average size. The width was approximately from 0.4 to 0.5  $\mu$ . They tapered slightly at both ends. There were from two to six spirals or more, the average being two or three. Under dark-field illumination they were found to possess a flagellum at each end, which was filamentary and two or three times as long as the body. Sometimes the flagellum was present at one end only. It was clearly seen in preparations made by Burri's method (Fig. 1), or impregnated with silver according to Levaditi (Fig. 2). The undulating membrane has not yet been observed.

*Staining of Spirochetes.*—The spirochetes stain uniformly, easily absorbing the color, when dyed according to Giemsa's method (Fig. 3) or with aniline dyes, or with Löffler's methylene blue, aniline gentian violet, or carbol-fuchsin, and they are Gram-negative. When the number of spirochetes contained in the blood is very small, the blood should first be thickly smeared over the cover-glass, then treated with 1 to 2 per cent acetic acid by Koch's method, and finally dyed with gentian violet. In one instance two spirochetes apparently in the process of division seemed to be joined together (Fig. 3). We shall report more fully on the morphology later.

*Experiments with Salvarsan.*

Fourteen inoculated white rats, in the blood of which spirochetes were observed, were divided into three groups and given subcutaneous injections of salvarsan. The groups received 0.2, 0.1, and 0.06 gm., respectively, per kilo of body weight. Although many spirochetes were present in their blood before inoculation, none were detected on the 1st, 2nd, 3rd, 6th, and 10th days after inoculation. On the 17th day only a small number of spirochetes was found in one of the white rats into which 0.06 gm. per kilo had been injected, while in the control rats they were constantly present. We also obtained the same result by injecting into thirty-six mice a quantity of salvarsan varying from  $\frac{1}{400}$  to  $\frac{1}{1,000}$  gm. per 20 gm. of body weight. The details of this experiment are shown in Tables I and II.

TABLE I.

*Injection of Salvarsan into White Rats.*

No. of animal.	Weight.	Dose per kg.	Spirochetes before injection.	Spirochetes after injection.							
				Day.							
				1	2	3	6	10	17	26	33
	gm.	gm.									
1	87	0.2	+++	-	-	-	-	-	-	-	-
2	78	0.2	+++	-	-	-	-	-	-	-	-
3	46	0.2	+++	-	-	-	-	-	-	-	-
4	53	0.2	+++	-	-	-	-	-	-	-	-
5	41	0.2	+++	-	-	-	-	-	-	-	-
6*	43	0.2	+++	++	+	+	+	+	+	+	+
7	43	0.2	+++	-	-	-	-	-	-	-	-
8	58	0.2	+++	-	-	-	-	-	-	-	-
9	172	0.1	+++	-	-	-	-	-	-	-	-
10	137	0.1	+++	-	-	-	-	-	-	-	-
11	59	0.1	+++	-	-	-	-	-	-	-	-
12	40	0.1	+++	-	-	-	-	-	-	-	-
13	115	0.06	+++	-	-	-	-	-	-	-	-
14	104	0.06	+++	-	-	-	-	-	+	-	-
15	77	-	+++	+++	+++	+++	+++	++	++	+	++
16	51	-	+++	+++	+++	+++	+++	++	++	+	++
17	40	-	+++	+++	+++	+++	+++	++	++	+	++

\* The dose of salvarsan was insufficient, owing to a technical mistake.

† Spontaneous decrease of spirochetes.



TABLE II.  
*Injection of Salvarsan into Mice.*

No. of animal.	Weight.	Dose per 20 gm.	Spirochetes before injection.	Spirochetes after injection.							
				Day.							
				1	2	3	4	7	10	14	17 19
	gm.	gm.									
1	15	$\frac{1}{400}$	++++	Died.							
2	15	$\frac{1}{400}$	++++	-	-	-	-	-	-	-	+
3	18	$\frac{1}{400}$	++++	-	-	-	-	-	-	-	+
4	15	$\frac{1}{400}$	++++	-	-	-	-	-	-	-	+
5	15	$\frac{1}{400}$	++++	-	-	-	-	-	-	-	+
6	15	$\frac{1}{400}$	++++	-	-	-	-	-	-	-	+
7	15	$\frac{1}{400}$	++++	-	-	-	-	-	-	+	+
8	15	$\frac{1}{400}$	++++	-	-	-	-	-	-	-	+
9	15	$\frac{1}{400}$	++++	-	-	-	-	Died.			
10	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
11	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
12	17	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
13	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
14	17	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
15	19	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
16	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
17	19	$\frac{1}{800}$	++++	-	-	-	-	-	-	+	+
18	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
19	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	+	+
20	17	$\frac{1}{800}$	++++	-	-	-	-	-	-	+	+
21	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	Died.	
22	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	+	+
23	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
24	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
25	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	+	+
26	15	$\frac{1}{800}$	++++	-	-	-	-	-	-	-	+
27	15	$\frac{1}{800}$	++++	-	-	-	-	-	+	+	+
28	15	$\frac{1}{1000}$	++++	-	+	-	+	+	+	+	+
29	17	$\frac{1}{1000}$	++++	-	+	+	+	+	+	+	+
30	15	$\frac{1}{1000}$	++++	-	-	-	-	-	-	+	+
31	17	$\frac{1}{1000}$	++++	-	-	-	-	-	-	-	+
32	15	$\frac{1}{1000}$	++++	+	-	-	-	+	+	-	+
33	15	$\frac{1}{1000}$	++++	+	-	-	-	-	-	+	+
34	17	$\frac{1}{1000}$	++++	+	-	-	-	+	+	+	+
35	18	$\frac{1}{1000}$	++++	+	-	-	-	+	+	+	+
36	17	$\frac{1}{1000}$	++++	-	-	-	-	-	-	+	+
37	15	-	++++	++++	-	++	++	+	+	+	+
38	18	-	++++	+	Died.						
39	13	-	++++	++++	++++	+	+	+	+	+	+
40	17	-	++++	++	++	+	+	+	++	+	+
41	15	-	++++	++++	++++	++	+	+	+	+	+
42	17	-	++++	++++	++++	+	++	+	+	Died.	
43	15	-	++++	++	++	+	++	++	++	+	+
44	17	-	++++	++++	++++	+	+	++	++	+	+
45	15	-	++++	++	++	+	+	+	+	+	+
46	15	-	++++	++++	++++	+	+	+	+	+	+

Controls.



*Identification with Other Species.*

Pathogenic and non-pathogenic spirochetes have been observed in many animals. We shall compare the various spirochetes with ours—except those which clearly differ in form—the animals in which they exist, and the results of experiments on animals.

(1) Wenyon's *Spirochæta muris*<sup>9</sup> is extremely motile. He attributes this to the flagella, though he does not claim to have proved it. The undulating membrane was not observed. The number of spirals varies between six and two according to the size of the spirochetes, which measure 6 to 7, or 3 to 4  $\mu$  in length and 0.2  $\mu$  in breadth. When inoculated into mice, they begin to appear in the blood in 5 or 6 days, and though few at first, they attain the greatest number about the 10th day and then decrease again in number. In 2 or 3 months they disappear entirely from the blood and at no period do they produce pathogenic symptoms in the mice.

Wenyon's spirochete and that found by us are similar in form, motion, the absence of pathogenicity for mice, and the manner of multiplication. In his experiment, however, guinea pigs did not become infected even with the whole blood of a mouse—a point on which Wenyon's spirochete differs from ours, since our spirochete invariably infects a guinea pig and can be seen in its blood. Wenyon further states that his spirochete can infect young rats but not full grown ones. Our spirochete, on the other hand, infects both young and adult rats.

(2) Breinl and Kinghorn's<sup>10</sup> *Spirochæta laverani* was found in a white mouse infected with *Trypanosoma dimorphon* sent by Laveran from the Pasteur Institute in Paris, and in the blood of two of the six wild mice caught in the neighborhood of the Liverpool School of Tropical Medicine. It is very motile, readily stains with the aniline dyes, and all the modifications of Romanowsky's method. It is generally short and round, but some are long, measuring between 1.8 and 3.75  $\mu$  in length and 0.1 and 0.2  $\mu$  in breadth. The number of spirals varies between two and four. Only a few of these parasites are found in the blood. It readily infects mice and rats, but gives rise to no pathogenic symptoms. Breinl and Kinghorn did not find many of the spirochetes in the blood, but could prove their existence in the peripheral blood 2 months after inoculation. They regard their spirochete as a distinct variety and have named it *Spirochæta laverani*. It seems to resemble our spirochete in form, movement, staining reaction, and its condition in mice and rats, but the result of the attempts to transmit it to guinea pigs is unknown.

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<sup>9</sup> Wenyon, C. M., *J. Hyg.*, 1906, vi, 580.

<sup>10</sup> Breinl, A., and Kinghorn, A., *Lancet*, 1906, ii, 651.

(3) Several investigators have found spirochetes in the cancer of mice. Borrel,<sup>11</sup> who was probably the first to do so, observed them in four mice suffering from primary cancers. After him Gaylord<sup>12</sup> examined forty-eight mice with cancer and more without cancer, chiefly by Levaditi's method, and observed numerous spirochetes. According to him, they stain readily by Levaditi's method, but not by Giemsa's; they are motile, but no flagella are seen; they vary between 2.5 and 7.8  $\mu$  in length, and have from four to thirteen spirals. Calkins<sup>13</sup> and Tyzzer<sup>14</sup> have reported the same spirochete.

Deetjen<sup>15</sup> likewise made a study of mouse cancer and observed a species of highly motile spirochetes. It stained readily by Giemsa's method, measured from 1.5 to 5  $\mu$ , and had from one to five spirals and a flagellum about 3.0  $\mu$  long at each end. He states that the flagella occasionally failed to stain except by Levaditi's method.

Löwenthal<sup>16</sup> observed small spirochetes from 2.6 to 6  $\mu$  in length and 0.25 or 0.2  $\mu$  in breadth with four to twelve spirals in ulcerated cancer from human beings and dogs. They stained readily; no flagella and no undulating membranes were seen, but Löwenthal believes that they are provided with the latter. He called this species *Spirochæta microgyrata*.

It is difficult to identify the species of spirochetes, since the size of a spirochete or the number of its spirals varies according to the method of treatment. In experiments on animals, too, different results do not prove conclusively the difference of species. Deetjen, however, thinks that his spirochete is identical with Wenyon's *Spirochæta muris* and Breinl and Kinghorn's *Spirochæta laverani*, and that although it is difficult to identify the spirochetes found by Borrel, and Gaylord, and Tyzzer with his, they are undoubtedly similar species. Wenyon, on the other hand, considers that his species is distinct from Carter's.<sup>17</sup>

We have examined the blood of a large number of normal mice and white rats, treated with acetic acid, but have not found a single spirochete. We have not, however, examined the cancer of mice; we cannot, therefore, attempt to identify our spirochete with those observed by the investigators mentioned above.

<sup>11</sup> Borrel, A., *Compt. rend. Soc. biol.*, 1905, lviii, 770.

<sup>12</sup> Gaylord, *Ann. Rep. Cancer Lab. New York State Dept. Health*, 1907, viii, 34.

<sup>13</sup> Calkins, G. N., and Clowes, G., *J. Infect. Dis.*, 1905, ii, 555.

<sup>14</sup> Tyzzer, E. E., *Proc. Soc. Exp. Biol. and Med.*, 1906-07, iv, 85.

<sup>15</sup> Deetjen, H., *Münch. med. Woch.*, 1908, lv, 1167.

<sup>16</sup> Löwenthal, W., *Berl. klin. Woch.*, 1906, xliii, 283.

<sup>17</sup> Carter, V., *Sc. Mem., Med. Officers Army of India*, 1887, iii, 45.

We should mention also the spirochetes in rats found by Carter<sup>17</sup> (1887), MacNeal<sup>18</sup> (1907), and Mezinescu<sup>19</sup> (1909). As we have stated, Wenyon finds it impossible to identify his spirochete with Carter's, and Breinl and Kinghorn also believe that Carter's spirochete, which is larger and has more spirals, must be different from Wenyon's. Mühlens,<sup>20</sup> however, is inclined to believe that they all belong to the same species. In only one instance, among all the normal rats whose blood we have examined, did we find spirochetes similar in form. Since we made no further test, we cannot, however, go into the details of the subject. The spirochetes found by MacNeal, Mezinescu, and others do not give rise to pathogenic symptoms in the animals into which they are inoculated. However, the spirochetes of these investigators and the spirochete found by us appear from inoculation tests to be similar in form. As their tests were not the same as ours, it is difficult to compare the spirochetes. Unlike the investigators referred to above, we first undertook the bite experiments and observed the course of the infection after the bite and the pathological appearance at autopsy, and finally the pathogenicity of the virus for the guinea pig and monkey. The favorable reaction with salvarsan seems also to be important.

As we have stated, we always observed traces of acute changes in the kidneys and adrenals of the guinea pigs bitten by rats and of guinea pigs inoculated from the original generation. We are, however, at present unable to compare experimental and human rat-bite fever since only two reports on the anatomical view of the latter have appeared. Blake believes that Schottmüller's *Streptothrix muris rattii* is the causative agent of the disease. His diagnosis was "acute ulcerative endocarditis; subacute myocarditis; subacute interstitial hepatitis; subacute glomerular and interstitial nephritis, subacute perivascular exudate of adrenals; infarcts of spleen and kidney; congestion, hemorrhage, and edema of lungs; atrophic leiomyoma of the uterus."<sup>21</sup> Blake's report, however, affords us little help in our anatomical study of guinea pigs, inasmuch as we cannot be certain whether the abnormalities in the kidneys and the adrenals were due to rat-bite fever or to a streptothrix. The other report, which is by Miura and Toriyama,

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<sup>18</sup> MacNeal, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1907, iv, 125.

<sup>19</sup> Mezinescu, D., *Compt. rend. Soc. biol.*, 1909, lxvi, 58.

<sup>20</sup> Mühlens, P., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, vii, 921.

<sup>21</sup> Blake, *J. Exp. Med.*, 1916, xxiii, 47.

does not mention the condition of the kidneys and adrenals. Crohn<sup>22</sup> described fifty-two cases of human rat-bite fever, and although he did not have the result of the examination of urine in every case, he enumerates nine cases of nephritis. From this it seems to be evident that human rat-bite fever also frequently affects the kidneys.

#### SUMMARY.

1. We have confirmed Ogata's results in experimental rat-bite fever caused by the bite of rats.

2. In our experiments with guinea pigs, swelling and congestion of the bitten parts, swelling of the subcutaneous lymph nodes, fever, and loss of weight were the typical symptoms. The progress of the fever was not so regular as in human cases, but we find records in the literature of patients who showed irregular fever types or were afebrile. The chief points that we noted in the anatomical view of the guinea pigs were swelling and congestion of the lymph gland system and acute changes in the adrenals and kidneys.

3. If an emulsion made from the lymph glands, cerebral substance, or the adrenals, or the heart's blood of a guinea pig of the original generation is inoculated subcutaneously or intraperitoneally into a fresh guinea pig, the animal invariably dies with the usual symptoms of fever and swelling of the lymph glands. The anatomical changes in this case were the same as those of the original guinea pig, except that the course was shorter and more regular. The same result was observed in further generations. No change was observed in pathogenicity, and in the guinea pigs of the original and further generations a species of spirochete as the causative agent was always observed. The incubation period in the original generation was from 1 to 2 weeks, and in further generations about 1 week.

4. When a mouse or white rat was inoculated, spirochetes always appeared in the peripheral blood, but no other symptoms developed. When peripheral blood drawn from a mouse thus treated was inoculated into a fresh mouse or a fresh white rat and peripheral blood drawn from the mouse and rat was inoculated into a fresh guinea pig, they all became infected and the guinea pigs always died. Thus we found that rats and mice are media but not victims of the disease, while guinea pigs are both media and victims of it.

<sup>22</sup> Crohn, B. B., *Arch. Int. Med.*, 1915, xv, 1014.

5. In the *rhesus* monkey on which we made our experiment we witnessed a process similar to that of human rat-bite fever, and our spirochetes were observed in other animals into which blood drawn from the monkey was inoculated.

6. In the original animals spirochetes were seen chiefly toward the end of the process and the conditions as to the period previous to it are not yet clearly known. In further generations of all the animals we used, spirochetes were found in the peripheral blood 4 or 5 days after inoculation, and gradually multiplied until the greatest number was reached about the 10th day after inoculation. They then began to decrease; yet spirochetes could be observed over 2 months later.

7. We have found spirochetes chiefly in the adrenals of the animals by Levaditi's method, but have not yet ascertained their distribution in other organs.

8. Our spirochete is short, round, and highly motile; it stains readily, and has few spirals. We have not yet observed an undulating membrane, but have seen what we believe to be a flagellum at each end.

9. The identification of our spirochete with other species must be left for further study. The spirochete which Futaki, Takaki, Taniguchi, and Osumi<sup>8</sup> found in two patients with rat-bite fever, seems to differ from ours in form.

10. Spirochetes disappear from the blood of the animals as a result of the injection of salvarsan, thus indicating that the spirochete is arsenotropic.

In conclusion we wish to express our indebtedness to Professor M. Ogata, to Professor Aoyama, Director of the Imperial Institute for Infectious Diseases, and to Professor Hayashi and Drs. Miyagawa and Mitamura, for their valuable assistance.

#### EXPLANATION OF PLATES.

##### PLATE 11.

FIG. 1. Spirochetes in the blood of a guinea pig infected with experimental rat-bite fever. Stained by Burri's method.

FIG. 2. A spirochete in a section of the adrenal of the same guinea pig as in FIG. 1. Stained by Levaditi's method.

FIG. 3. Spirochetes in the blood of a guinea pig infected with experimental rat-bite fever. Giemsa's stain. Zeiss, oc. 4, obj.  $\frac{1}{2}$  oil immersion.





FIG. 1.

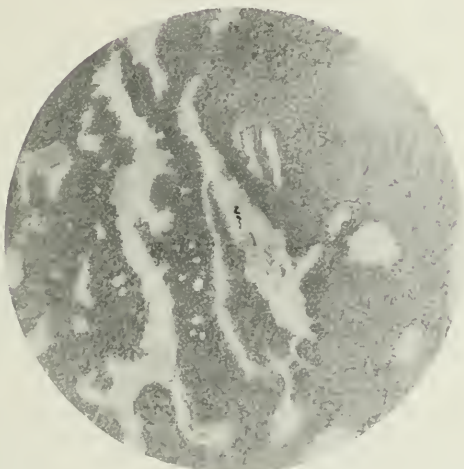


FIG. 2.



FIG. 3.

(Ishiwara, Ohtawara, and Tamura: Experimental Rat-Bite Fever.)





## THE ACTION OF DIGITALIS IN PNEUMONIA.

By ALFRED E. COHN, M.D., AND ROSS A. JAMIESON, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATE 12.

(Received for publication, October 17, 1916.)

Digitalis has been used for many years in the treatment of pneumonia but there is still discussion as to whether its use is advantageous. A decision has been difficult because the difference between action as such and beneficial action has not been sharply drawn. We show in this paper that action on the heart by digitalis takes place in pneumonia, and also that its action under certain circumstances is beneficial.

### HISTORICAL.

Schmiedeberg (1) has given the early history of the use of digitalis in pneumonia. It was recommended by Ferrier (1799) for the purpose of slowing the heart rate instead of resorting to phlebotomy. Currie (1804) preferred it to cold water treatment in cerebral, cardiac, and pulmonary inflammations. Cumming (1804) gave it after phlebotomy. Then, says Schmiedeberg, its use was almost forgotten. Traube (1850), who learned the value of digitalis in Schoenlein's clinic, reintroduced it. Because it slowed the pulse Traube believed that it was an antipyretic but, as von Leyden showed, he failed to recognize the fact that if the pulse rate was slowed to the point of lowering temperature, this result was due to collapse. At this point Schmiedeberg leaves the history. He believed that giving digitalis in pneumonia was a matter still open to investigation.

More recently clinicians have been divided in opinion on its usefulness in this disease. Gibson (2) is doubtful; and Mackenzie (3) says: "I have never seen much good follow the administration of digitalis in acute febrile states. The factors exciting the heart, such as high temperature, toxins, or the invasion of the heart by specific organisms, exert an influence over the heart which the digitalis can not overcome." Lauder Brunton (4), in discussing the effect of temperature (not fever) on the action of drugs goes so far as to say: "Perhaps one of the most marked examples of this is digitalis, which at a high temperature completely loses its power of slowing the heart through the vagus." In the United States, certain authors, as Hare (5), share Brunton's view. Others, like

Elsner (6), enumerate specific indications for giving digitalis. In Germany, on the other hand, digitalis is more commonly administered now. Romberg (7) gives it to all pneumonia patients suffering from heart disease as soon as the diagnosis is made. Meyer (8) reports that it is constantly used in Krehl's clinic with gratifying results. Fraenkel (9) gives large doses (3 to 4 gm. a day for the first 3 days) in accordance with the Traube tradition.

An effort has been made to ascertain by experiment whether in the presence of fever any agents are at work which interfere with the action of digitalis. All these experiments have been employed for the purpose of determining whether heat interferes with the action of the drug. Trendelenburg (10), Weizsäcker (11), Gunn (12), and von Issekutz (13) have all performed such experiments either on rabbit or frog hearts, with digitalis or amorphous or crystalline strophanthine preparations. The result of all these efforts has been to show that by elevating the temperature of the perfusion fluid containing the digitalis body, the end-point of the reaction appears earlier than in the controls; that, in other words, elevation of temperature facilitates the action of digitalis bodies.

In the experiments performed by Jamieson (14) on dogs and cats, on the other hand, the problem investigated was whether pneumonic infection had an influence on the action of digitalis other than that exerted by temperature alone. He could find no difference in the minimal lethal dose of crystalline *g*-strophanthine in normal animals and in infected animals when the infection was at its height. He concluded, therefore, that there was nothing in the nature of the infection to interfere with the action of the drug. This conclusion does not, however, mean that the heart in pneumonia is an altogether unchanged and intact organ. Newburgh and Porter (15), for instance, have shown that it must be altered, for the hearts of animals infected with the Friedländer bacillus continue to beat when nourished with pneumonic blood, whereas such blood quickly poisons control hearts from non-infected animals. It is clear, however, from the results of Jamieson's work that, whatever the poison to which pneumonic hearts have accommodated themselves, its nature is not such as to interfere with digitalis activity.

### *Method.*

Our observations have been carried out on 105 cases of pneumonia treated in the Hospital of The Rockefeller Institute. Digitalis was given to 49 patients; the other 56 received no digitalis and served as controls. The drug was usually given by mouth in the form of tablets of digipuratum, each tablet containing the equivalent of 0.1 gm. of the powdered leaves. The daily dose was usually 0.4 gm. Electrocardiograms were made of all the patients. In the patients to whom digitalis was administered the curves were made frequently, usually once, or oftener, each day. At the beginning of the study,

TABLE I.

	Control. No digitalis.			With digitalis.					Total available.
	Repeated records.	Single records.	Total.	Repeated records.		Single records. Not available.	Totals.		
				Available.	Not available.				
Recovered.....	41	8	49	28	3	—	31	77	80
Died.....	4	3	7 (12.5%)	11 (28.2%)	3	4	18 (36.7%)	18	25 (18.9%)
Total.....	45	11	56	39	6	4	49	95	105

### *Detail of Cases.*

	Fever.		No fever.	Per cent.		Fever.		No fever.	Fever.		No fever.	Total. Per cent.		Available. Per cent.	
	Recovered.	Died.		Fever and no fever.	Fever.	Recovered.	Died.		Fever and no fever.	Fever.		Recovered.	Died.	Fever and no fever.	Fever.
Changes P-R and T.	2			3.5	4.0	21	7	2				61.2	60.8	76.9	77.77
“ T alone.	3	1	4	16.0	8.0	3	3				2	22.4	23.9	15.3	16.66
“ P-R						1	1	1			1	4.08	2.1	5.1	2.77
“ neither.	32	3		80.3	88.0		1				2	12.2	13.04	2.5	2.77
Totals.....	37	4	4	56	50	25	11	3			4	49	46	39	36
	45			11			39			6			4		
													97.2		

however, daily electrocardiograms were not made because the importance of frequent observations for the purpose of this investigation was not appreciated.

We have utilized as criteria for judging the action of digitalis the effect upon the length of the auriculoventricular interval (the P-R time) and the effect on the T wave of the electrocardiogram. The length of the auriculoventricular interval was calculated from electrocardiograms. The changes in the T wave for which we looked were the ones described in an earlier paper (16). In certain cases the effect of digitalis on the rate of the ventricles when the auricles were fibrillating has been an important additional criterion.

#### RESULTS.

In general the criteria we employed permitted us to judge satisfactorily whether digitalis was acting. We found that the signs appeared after the same amount had been given and following the same length of time in which these signs appeared in the non-febrile cases originally studied. When no digitalis was given the signs did not appear.

In 45 of the 56 control cases (Table I) we were able to make two or more observations (repeated records), while in 11 others only single electrocardiograms (single records) were obtained.

Of the 49 patients to whom digitalis was given, 39 are represented by two or more electrocardiograms (repeated records). They are grouped as available because they received amounts of digitalis sufficient to influence the curve. 6 patients are classified as unavailable because they took total amounts of digitalis (from 0.3 to 0.6 gm.) which as a matter of experience are regarded as too small to influence the curve. The remaining 4, on whom only single observations were made, are rejected for the same reason. The doses given to them were 0.7, 0.1, 0.4, and 0.2 gm.<sup>1</sup> The amount regarded as sufficient to produce the changes we looked for was fixed at 0.8 gm. No one showed changes after taking an amount less than this, except a single patient to whom the drug may have been given before admission to the hospital.

<sup>1</sup> In the last three cases the amounts given are approximate.

We distinguish further, both in the control and in the digitalis groups, between those patients who showed alterations in the curves during fever and those in whom defervescence had already taken place when the alterations were found. This distinction is necessary because what we wish to ascertain is the action of digitalis during the continuance of fever. The cases have been further subdivided into (1) those which showed changes both in P-R time (auriculoventricular interval) and in the T wave; (2) those which showed changes in the T wave alone; (3) those which showed changes in the P-R time alone; and (4) those which showed changes neither in the P-R time nor in the T wave. We base our percentage figures, therefore, on 50 cases of pneumonia who received no digitalis during the febrile period of the disease; and on 36 to whom digitalis was administered, and who took a sufficient amount of the drug.

Of the 50 control patients, 88 per cent (44 cases) showed no change in either P-R time or in the T wave; 8 per cent (4 cases) showed changes in the T wave alone; 4 per cent (2 cases) showed changes in both the P-R time and in the T wave. Of the 36 patients to whom digitalis was given, on the other hand, 77.7 per cent (28 cases) showed changes both in the P-R time and in the T waves; 16.6 per cent (6 cases) showed changes in the T wave alone; 2.7 per cent (1 case) showed changes in the P-R time alone; and 2.7 per cent (1 case) showed changes in neither the P-R time nor in the T wave. The results show briefly, therefore, that during the febrile period 88 per cent of the control cases showed no change in the electrocardiogram; while 97.2 per cent of the cases to whom digitalis was given, did show changes.

Besides the effect on conduction and on the form of the electrocardiogram (T wave), we were able to study the action of digitalis on the rate of the ventricles when the auricles were fibrillating. In five separate instances when this rhythm set in, digitalis reduced the resulting high ventricular rate while fever was present. These cases will be described in detail in a separate communication.

The facts given permit us to conclude that digitalis acts in pneumonia.

*Exceptions.*—The cases here described are those which were not included in the two percentage figures (88 and 97.2 per cent) just given. These include



6 control cases during fever, 5 others after defervescence, and 4 (1 febrile and 3 non-febrile) available and 10 non-available cases to which digitalis was given. We shall take up first the 6 control cases during fever. 2 showed changes both in the P-R time and in the T wave. It cannot be definitely stated that these changes occurred spontaneously, because it is not known whether digitalis was given outside the hospital. 4 other control patients showed changes in the T wave alone. 2 of these (a mother and her son) entered the hospital rather late in the course of the disease (the son on the 5th day). The 3rd showed the change only in the first curve; the 4th in a curve a week before death, on the 4th day in the hospital. In addition to these, 5 other control patients showed changes in the T wave in the period after defervescence. We can offer no explanation for their occurrence. The point of immediate interest lies in the fact that when no digitalis was given, the occurrence of electrocardiographic changes during fever was infrequent (6 in 49 cases).

We take next the single patient to whom a sufficient amount of digitalis was given and in whose curves during the febrile period the expected alterations did not take place. This patient died. Three curves were made in the 3 days he was in the hospital, and all showed that the T wave in Lead I was inverted. Whether this was normal for him, or whether it represented a change is unknown. No further change in his curves was observed, and we classify him accordingly as having shown none. In the other three available cases it is necessary only to notice that the changes occurred after the fever ceased.

We consider finally the 10 unavailable cases to which digitalis was given during fever. 5 showed changes in the T wave alone; 5 showed changes in neither. In the latter 5 no change is expected (they had taken insufficient amounts); they require no further discussion. The other 5 took digitalis, but in insufficient amounts. Of these, 1 recovered and 4 died. The one who recovered took 0.5 gm. before the electrocardiogram changed. After convalescence his T wave again became upright. The 4 who died took, respectively, 0.6 gm., 0.5 gm., 0.2 gm., and the last probably less than 0.5 gm. (The time when the electrocardiogram was taken is not recorded.) Whether doses so small caused the change must remain undecided. We doubt that the drug can have been the cause. In these 5 cases to which digitalis was given and in the 6 (febrile) control cases, then, changes took place when no digitalis had been administered, at least in the hospital, or when it was given in amounts insufficient to satisfy one that digitalis was responsible for the change.

Although our report deals with the changes which occur primarily during fever, we have included in separate columns in the table those cases in which changes took place in the postfebrile period, and we have given the percentages of each variety of change in the total of febrile and non-febrile cases. In the cases to which digitalis was given the total available and the total non-available cases are given separately. The general interpretation we draw from our observations is not altered by this division. The conclusion that digitalis acts on the febrile heart in pneumonia is, of course, better seen when only the available cases in each group are considered.



## DISCUSSION.

*Auriculoventricular Conduction (P-R) Time.*—The effect of pneumonia or the intoxication of pneumonia on heart muscle has generally been supposed to bring about an increase in the difficulty of auriculoventricular conduction. A number of cases have indeed been reported in which delay in, or block to the passage of impulses has occurred. But in the 50 control cases on which we report, it is striking that a significant increase in conduction did not occur. On the contrary, we observed an opposite tendency with equal frequency. A decrease in the conduction time was observed in 11 cases (Table II). The decrease was, however, slight; it was usually 0.02 to 0.03 second, twice 0.04 second, once 0.05 second, and once 0.06 second. When

TABLE II.

Hospital No.	P-R time.
2,315	Fell from 0.16—0.14 = 0.02 sec.
2,309	“ “ 0.16—0.13 = 0.03 “
2,270	“ “ 0.17—0.14 = 0.03 “
2,276	“ “ 0.20—0.17 = 0.03 “
2,325	“ “ 0.17—0.14 = 0.03 “
588	“ “ 0.18—0.15 = 0.03 “
2,187	“ “ 0.16—0.13 = 0.03 “
2,286	“ “ 0.17—0.13 = 0.04 “
2,202	“ “ 0.18—0.14 = 0.04 “
2,195	“ “ 0.18—0.13 = 0.05 “
2,269	“ “ 0.21—0.15 = 0.06 “

lengthening took place, as it did in 6 patients, the change occurred within limits as narrow (Table III) and did not extend beyond 0.3 second.

TABLE III.

Hospital No.	P-R time.
2,335	Rose from 0.15—0.17 = 0.02 sec. during fever.
2,296	“ “ 0.14—0.16 = 0.02 “ “ “
2,344	“ “ 0.14—0.17 = 0.03 “ “ “
2,184	“ “ 0.17—0.20 = 0.03 “ “ “
2,216	“ “ 0.13—0.16 = 0.03 “ after “
2,168	“ “ 0.15—0.18 = 0.03 “ “ “

The absence of lengthening in conduction leading to heart block in this series<sup>2</sup> is the more striking on account of the fact that we are taught to look for its occurrence in the course of infectious fevers. In our control cases, as we have shown, it did not take place.<sup>3</sup> In the cases to which digitalis was given, on the other hand, there was with only three exceptions an increase of 0.04 second or more. In seven instances the rise led to block.

It is conceivable that delayed conduction in patients with pneumonia may occur simply as the result of the increased rate of the pacemaker; and second, that it may be a direct effect of the intoxication due to pneumonia itself. As far as the effect of increased rate is concerned, Lewis and Cotton (17) have shown satisfactorily that when normal individuals exercise, no lesion of the junctional tissues being present, the hearts respond by an increase in rate and a decrease in the auriculoventricular interval of 0.01 to 0.03 second. In our patients the conduction interval tended, indeed, during the height of the fever, to be lessened. This may have been due to the elevation in rate. When lengthening occurred it was distinctly within the range found in normal hearts. The influence which the intoxication of pneumonia exerts on this function will be discussed later.

A reasonable explanation for the failure of a marked change in conduction to occur spontaneously may be found in a consideration of the histological pathology of pneumonia. The infections which have an influence on auriculoventricular conduction are acute rheumatism, diphtheria, typhoid fever, syphilis, and occasionally influenza. The mechanism which brings this effect about in these diseases probably does not exist in pneumonia. In the other infections mentioned, definite anatomical changes may take place, as, for instance, focal necroses in typhoid fever; infiltrating and destructive

<sup>2</sup> In another series of patients, now being studied, an irregularity occurred in the postfebrile period in one case caused by premature auricular beats, which were blocked. These occurred during the systole of the preceding ventricle. The ventricle may have been in its refractory period. The conduction time was uniformly 0.14 second. Incomplete block in the ordinary sense was therefore absent.

<sup>3</sup> The conduction changes reported by others did not, except rarely, occur until a sufficient dose of digitalis had been administered. This subject will be discussed in a later paper.

inflammatory invasions in rheumatism and syphilis; fatty, granular, and waxy degeneration of the muscle and interstitial round cell infiltration in diphtheria. Any one of these alterations may involve the conduction pathway and may terminate in functional disturbance. Of the pathological histology of the heart in pneumonia there is still insufficient knowledge, but it does not appear that extensive alterations occur.

Hearts from pneumonia patients have been described by Romberg, Meyer, Aschoff and Tawara, Eyslein, Zadek, Thorel, Fraenkel, and Liebmann. In a recent review of the literature, Thorel<sup>4</sup> (18) says: that from the point of view of pathological anatomy the changes in the heart in pneumonia are insignificant. Serious myodegenerations do not occur. Fatty degeneration is occasionally seen in older individuals. Romberg and his associates (19), in experiments on animals, were struck by the absence even of a slight effect on the behavior of the heart in pneumococcal infections. They saw only small and unimportant changes in the heart muscle.<sup>5</sup> Zadek (20) found no lesion in one case and myodegeneration in another case (the second had slight albuminuria) dying late in convalescence. Aschoff and Tawara (21) examined five cases and could find no myocardial process or extensive specific parenchymatous change. Liebmann (22) in a more than ordinarily careful examination of eleven hearts, found lesions in only three, but in these three he found inflammatory infiltration. Such lesions in suitable locations might cause disturbances in rhythm. Eyslein (23) examined twenty-three cases; nineteen hearts showed no particular changes, four showed some fatty change, and three of these an advanced degree of this alteration (Table IV).

TABLE IV.

Author.	Total.	Lesions.
Zadek.....	2 cases.	1 case.
Aschoff and Tawara.....	5 "	0 cases.
Liebmann.....	11 "	3 "
Eyslein.....	23 "	4 "
	41 "	8 "

Anatomical lesions are, according to these investigations, infrequent and on the whole insignificant. This conclusion strengthens the belief, formed by clinical observation, that where moderately

<sup>4</sup> Thorel (18), p. 432.

<sup>5</sup> Romberg, Pässler, Bruhns, and Müller (19), p. 713.

lengthened conduction occurs, it is not due to an anatomical cause. Whatever the nature of the agent, it is clear that its action is not uniform, for within the narrow limits in which changes in our series took place, they were, as we have shown, in the direction both of lengthening and shortening.

*T Wave.*—In our earlier paper (16) we suggested that the occurrence of the T wave phenomenon was probably explained by the fact that since "cardiac action currents depend on electrical changes as an expression of muscular activity, then the changes in the T wave must be attributed to an alteration in muscular state under the influence of the drug" (digitalis). We pointed out at the same time that the change in the T wave we were describing was not specific for digitalis, but that it occurred under the influence of muscarin, under the alteration of physical states (as the application to the heart of heat and cold), and as the result of stimulating the vagus nerve by interrupted electrical currents. There is a temptation to add the intoxicating substance in pneumonia to the agents able to cause it, in view of the fact that a number of untreated cases showed a change in the T wave. But we do not include it because the change occurs so infrequently and because of the possibility that patients may have been given digitalis before being brought to the hospital. Furthermore, the T wave in nine control cases suffered no decrease in amplitude, but actually increased distinctly (Fig. 1). When to these considerations is added the fact that the change occurred in five patients after defervescence or after the crisis, then it becomes improbable that the intoxicating agent in pneumonia possesses this influence. What circumstance is responsible for the unexpected changes which we found we are not able to decide.

We shall report next on the dose required, and the order in which the effects on the T wave and on conduction take place. It is impossible to decide on the smallest efficient dose. That was possible in the non-febrile cases described in our earlier report, where there was no complicating intoxication, and where one could be sure that no digitalis had been given before treatment. The dose we regarded as effective was about 1 gm. To the pneumonia cases we have given almost uniformly 0.4 gm. in 24 hours. The largest total doses we gave were 4.45 gm. (1 patient), 3.8 gm. (1 patient), 3.3. gm. (1 pa-

tient), 3 gm. (1 patient), and 2.9 gm. (1 patient). The other 44 patients took 2.5 gm. or less.

We found in the group of available cases that alterations in the T wave alone were observed in 16.6 per cent, and changes in the P-R time alone in only 2.7 per cent. Where only one of the changes takes place, that change accordingly is more likely to affect the T wave. But where both alterations were observed (28 cases, 77.7 per cent) we found that they occurred simultaneously in 15 patients, that the T wave was altered with smaller doses in 8 patients, and that the P-R interval lengthened earlier in 5 (Table V).

TABLE V.

Hospital No.	Change in P-R time.	Change in T wave.
	<i>gm.</i>	<i>gm.</i>
2,338	After 0.9	After 0 (—T before digitalis.)
2,381	" 1.8	" 0.8
2,208	" 1.2	" 0.9
2,277	" 3.4	" 1.1
1,991	" 1.7	" 1.3
2,247	" 3.0	" 1.5
1,858	" 2.4	" 1.6
2,287	" 2.6	" 2.3
2,413*	After 0.4	After 0.8
2,284	" 0.8	" 1.2 (No fever.)
2,222	" 1.1	" 1.5
2,175	" 1.3	" 1.5
2,399	" 1.3	" 1.7

\* This patient probably was given digitalis before admission to the hospital.

We conclude, therefore, that digitalis alters the T wave oftener and sooner than it does the P-R time. This result is in agreement with that found formerly by us when the results of giving digitalis to patients suffering from heart disease were reported. There, in all but five instances the changes in the T wave occurred before the change in conduction. In the present series, in all but five cases, the change in the T wave occurred at the same time with, or before the change in P-R time. As a guide, therefore, to giving digitalis to patients suffering from pneumonia, we ascribe a distinct value to the use of the T wave as a sign.



*Method of Selection.*—The frequency with which changes in P-R time and in the T wave occurred in the digitalis group might lead to the belief that, since in this group the mortality was high, the changes were due to approaching death. In other words, the great number of electrocardiographic changes in the cases to which digitalis was given may have been due either to the method of selection or to the influence on the heart exercised by the mechanism of approaching death.

The most satisfactory method of selection consists, obviously, in studying alternately control and treated cases. We did not adopt this method because we were unwilling to withhold digitalis from patients who were seriously ill. In the early cases, indeed, it was only the severe ones that were treated. They show, consequently, a larger number of deaths than would ordinarily be expected. The mortality of the control cases was low (Table I), 12.5 per cent (7 in 56 cases), but of the available treated cases it was more than twice as great, 28.2 per cent (11 in 39 cases). Of the untreated and available treated cases it was 18.9 per cent (18 in 95 cases). The average percentage of mortality in the hospital covering the period in which these cases were studied was 27.8 per cent (Table VI). That the

TABLE VI.

Year.	No. of cases examined.	Received digitalis.	Died.		Hospital mortality.
			No.	Per cent.	
		<i>per cent</i>			<i>per cent</i>
1911-12	6	50	4	66.6	47.6
1912-13	16	50	6	37.5	17.3
1913-14	19	68.4	8	42.1	20.2
1914-15	82	56.9	21	25.6	26.1
	123	56.3	39	42.9	27.8

mortality of the treated cases is high on account of the method of selection is suggested by the fact that in the year 1914-15, when we studied all the cases (with seven exceptions), we had the lowest mortality (25.6 per cent), whereas in the year 1911-12, when we studied only severe cases, the mortality was 66.6 per cent. The mortality of all the patients treated in the hospital during the same



TABLE VII.

*Relation of Time of Death to Time of Making Electrocardiogram.*

Case No.	Hospital No.	P-R + T.	T alone.	P-R alone.	No change.	Digitalis.		Time before death of		Time in hospital.
						Total.	Effective.	Change in electrocardiogram.	Last electrocardiogram.	
Without digitalis.										
1	2,305		+			gm.	gm.	7-0-0*	7- 0- 0	
2	1,918				0	0.4	0		1-12-39	
3	2,314				0	0.4	0		1- 1-20	1 curve
4	1,776				0		0		1- 0- 0	
5	2,157				0	0.4	0		0- 7-45	1 "
6	535				0		0		0- 5-10	Lead II only.
7	438				0		0		0-12-20	" II " 1 curve.
									2-5-25	
With digitalis.										
8	2,322†				0		0.7		0- 7-17	About 0-24-0 2 curves.
9	1,919†				0		0.7		0- 7-50	" 2-0-0 1 curve.
10	2,308				0		1.3		0- 1- 0	3 curves.
11	2,205†				0	0.3	0		0- 1- 0	About 0-24-0 1 curve.
12	1,899†		+				About 0.4		0-12- 0	1-23-2 1 curve.
13	2,231†		+			0.5	0.2		0- 8-50	About 1-4-55 1 curve.
14	2,339†		+			0.7	0.6	0-3-43	0- 3-43	1-4-13 2 curves.
15	2,355†		+			1.0	0.6	1-6-30		
16	2,268		+			0.9	0.8	0-4-50	0-4-50	About 2-2-20 3 curves.
17	1,963	+					1.2	0-8-0	0-8- 0	2 curves.
18	2,287	+				2.7	2.3	1-11-10		
19	2,295		+			1.6	0.9	1-21-8		
20	2,201		+			1.9	1.5	2-9-50		
21	2,230		+			2.2		3-0-0		
22	2,277	+	+				0	9-0-0		
				+		3.8	2.0	5-0-0		
23	2,208	+				1.3	1.1	6-0-0		
24	1,858	+	+				2.4	6-0-0		
				+		4.45	1.6	7-0-0		
25	2,247	+				3.3	1.1-1.5	12-0-0		

\* The numbers 7-0-0, etc. refer to days, hours, and minutes, respectively.

† Repeated records not available.

‡ Single records not available.

year was 47.6 per cent. The number is small to serve as a basis for generalization, but it is clear that the administration of digitalis had nothing to do with the high mortality rate, for in each year approximately one-half the patients observed took the drug.

If the number of electrocardiographic changes was due to the high rate of mortality among the treated cases, the ground for this must be sought in alterations in the heart occurring during the agonal period or in the period 1 or 2 days before death. If the changes occur in the heart at that time, a high rate of mortality would naturally increase their number. It is necessary, therefore, to decide whether these changes occur at or about the time of death. We have accordingly analyzed the curves of all the patients (25) that died.

10 cases (Nos. 2 to 11) showed no changes (Table VII). 7 (Nos. 2 to 7, and 11) of these had taken no digitalis, while of the 3 (Nos. 8, 9, and 10) who had, 2 (Nos. 8 and 9) took insufficient amounts; that is, 9 (Nos. 2 to 9, and 11) behaved in the expected way. Of the 15 (Nos. 1, and 12 to 25) that showed changes, 1 (No. 1) had taken no digitalis, and 4 (Nos. 12 to 15) had taken insufficient amounts. The remaining 10 cases require no separate consideration; they had all taken enough digitalis to account for the result. The results, in summary, show that 19 of the 25 (76 per cent) patients either showed no changes when digitalis was not given, or given in insufficient amounts, or showed changes when sufficient amounts had been taken. The last curves examined in cases in which there were no changes in the curves were all taken from 1 to 36 hours before death; all but four were taken within 8 hours, and two within 1 hour. The fact that so many died without changes must be taken to mean that the proximity of death was not associated with the electrocardiographic changes on which we rely, even in those who had taken small amounts of the drug.

From a consideration of all the evidence we have been able to gather, we believe that the intoxication due to pneumonia exerts no influence against the action of digitalis. Digitalis produces the same effects in pneumonia, judged by the criteria we use, that it does in the absence of fever. We believe ourselves justified in assuming, therefore, that whatever actions the drug possesses, are exercised also during pneumonia. If the action is beneficial, advantage

may be expected from its use. That other observers have seen no results from giving it during severe pneumonia may depend on the fact that unjust criteria were employed for estimating its action.

#### SUMMARY.

We have shown in a series of 105 cases of pneumonia, 95 of which we have selected as available for statistical study, that digitalis given by mouth has an action on the heart. We have judged this action to be present because changes occurred in the auriculoventricular conduction time and in the form of the T wave of the electrocardiogram, just as they do in the non-febrile heart. This conclusion is strengthened by finding that the pulse rate in fibrillating and fluttering cases fell in the presence of fever, exactly as it does in non-febrile cases. The dose and the time required to produce these effects are given and are the same as in the non-febrile cases. When there was a difference in the amount necessary to produce one or the other of the changes, it was found that the T wave is more often and more readily affected than the conduction interval. We have shown that the intoxication due to pneumonia is probably not responsible for the changes found, both from a study of the statistics and because in the control cases reverse tendencies were often found (that is, decrease in conduction time and increase in the size of the T wave). We have shown that the method of selection in consequence of which we treated a large number of severe cases did not prejudice our results, because it could be demonstrated that the proximity of death, whether in control or treated cases, was not necessarily associated with the changes we are describing. We have also, by referring to the literature of the subject, brought evidence to show that heart muscle does not undergo those changes in pneumonia, as it does in other infectious diseases, which would lead one to expect changes in conduction found in other diseases. The changes in conduction which have been reported by others were almost entirely associated with the giving of digitalis.

## CONCLUSIONS.

1. Digitalis acts during the febrile period of pneumonia.
2. It produces a beneficial, possibly a life-saving effect in cases of auricular irregularity (fibrillation and flutter).
3. Whatever beneficial action it has on the function of the normally beating non-febrile heart may be expected from its use in the febrile heart in pneumonia.

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## EXPLANATION OF PLATE 12.

Fig. 1. Divisions of the ordinates equal 0.1 millivolt. Divisions of the abscissæ equal 0.04 second. In each set of these electrocardiograms, Leads I, II, and III are arranged from above downward.

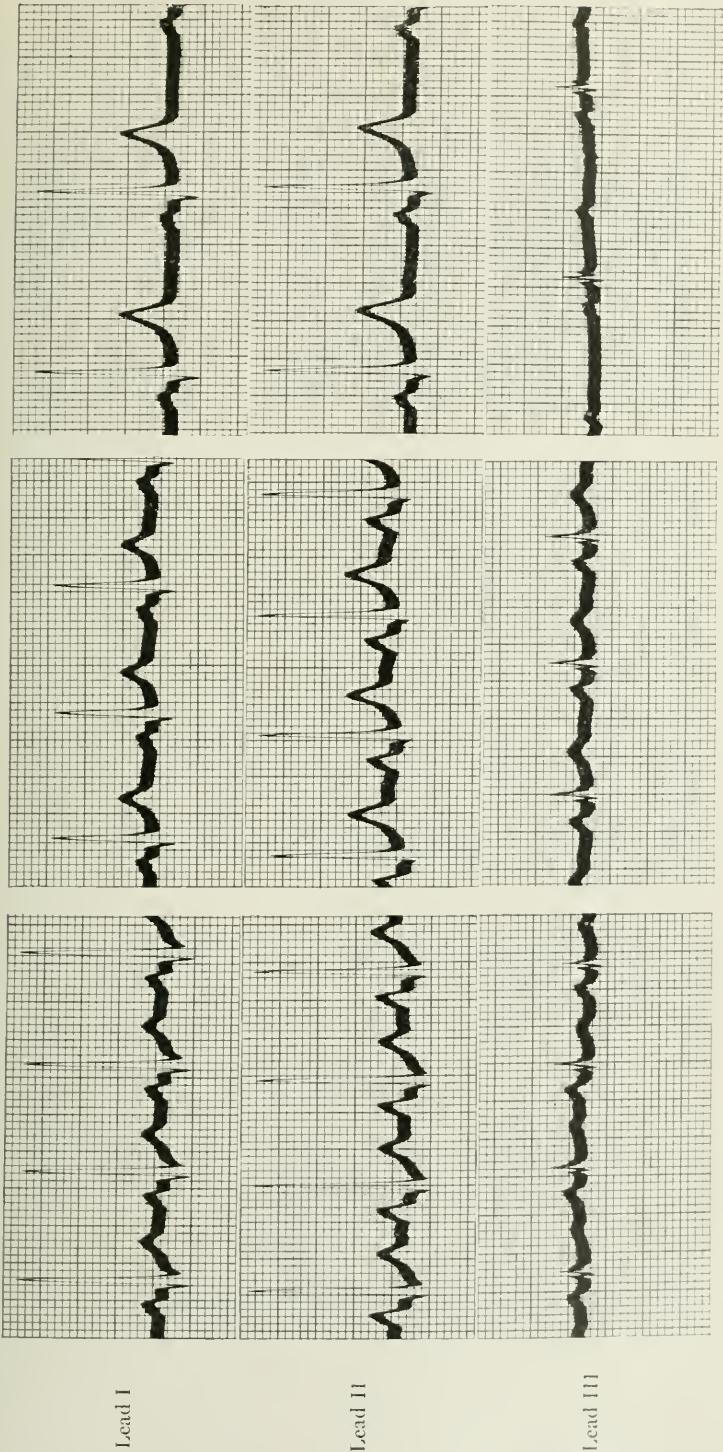
A. These electrocardiograms were taken on January 24, 1915, the 3rd day after the onset of pneumonia. The temperature on this day ranged between 103° and 104.6°F. The *P-R* time was 0.15 second.

B. These curves were taken on January 26. The temperature ranged between 102.2° and 103.6° F.  $T_1$ ,  $T_2$ , and  $T_3$  are taller than in A. The *P-R* time was 0.16 second.

C. These curves were taken on February 3 during convalescence.  $T_1$  and  $T_2$  are taller than in either A or B;  $T_3$  is less tall. The *R-T<sub>3</sub>* period is lengthened. The *P-R* time is 0.16 second. Apparently the  $T_1$  and  $T_2$  waves increased in size throughout the period of observation. The *P-R* time lengthened, and on February 28 was 0.21 second.







C

B

FIG. 1.

A

Lead I

Lead II

Lead III

(Cohn and Jamieson: Action of Digitalis in Pneumonia)



# THE CONTROL OF STRYCHNINE CONVULSIONS BY INTRASPINAL INJECTIONS OF MAGNESIUM SULPHATE.

By ELLIOTT C. CUTLER, M.D., AND BENJAMIN H. ALTON, M.D.

(From the Surgical Clinic of the Massachusetts General Hospital, Boston.)

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In cases of strychnine<sup>1</sup> poisoning, text-books on pharmacology and therapeutics (Kobert (1, 2), Tyrode (3), Cushny (4), and Dixon (5)), even of recent date, advocate evacuation and washing of the stomach by tube or by the usual emetics, accompanied by the administration of tannic acid or some other astringent, to remove the portion of the drug remaining in the stomach, and an attempt to control the convulsions by a depressant of the central nervous system, as urethane, chloral, bromides, ether, or chloroform. Kobert (2) advises, in addition, the use of small doses of curare, which must, however, be given with great care. Githens and Meltzer (6, 7) found that animals given a twice lethal dose of strychnine could invariably be saved by the use of intratracheal ether anesthesia plus the intravenous administration of considerable quantities of Ringer's solution. The success of their experiments apparently depended on the effect of Ringer's solution in hastening the excretion of the strychnine, the anesthesia merely controlling the convulsion until the drug was eliminated. Lethal doses were then rapidly excreted in the urine. The average duration of the anesthesia was  $4\frac{1}{2}$  hours.

The experiments of Githens and Meltzer were not known to us at the time of our study. Until now, little hope of recovery has been offered in cases where the convulsions have become general with opisthotonos, for respiratory paralysis soon follows.

## *Report of a Case of Strychnine Poisoning.*

The present study was undertaken to confirm the results obtained in the following case.

Baby C., female, 1 year and 2 weeks of age; weight 22.5 pounds. Full term, normal delivery; breast fed for 1 month; healthy; no pre-

<sup>1</sup> U. S. census report for 1909 (see Wilbert, M. I., Poisons and Habit-Forming Drugs, *Public Health Rep.*, No. 330, 1916), gives strychnine poisoning as the cause of death 210 times, in 113 instances suicidal, and in 97 instances accidental.

vious illness. Admitted to the Massachusetts General Hospital, Accident Ward, at 5.30 p.m., November 25, 1915. 1 hour before admission the child had swallowed 12 to 14 pills which its father was taking for stomach trouble. The prescription showed these to contain  $\frac{1}{30}$  of a grain of arsenic,  $\frac{1}{60}$  of a grain of strychnine, and 1 grain of iron.

The mother gave the following history. 15 minutes previously the child stopped playing and had a slight general convulsion. It vomited once shortly after. The convulsive seizures were repeated in rapid sequence and the intensity increased. By the time the hospital was reached, the child was cyanotic and in an almost continuous convulsion, in which opisthotonos was frequent, with periods of total respiratory inhibition.

Ether was at once administered and the stomach tube passed. In the vomitus coming about the tube, and in the stomach washings three pills and several fragments of pills were recovered. Sodium bromide, 45 grains, was given by rectum but was at once expelled. The dose was repeated again but again the bowels moved. The same amount of the drug was administered by stomach tube but was later vomited. Urine was passed spontaneously. The color was better under ether. Twice the anesthesia was removed, but the child on both occasions had severe general convulsive seizures with opisthotonos and respiratory inhibition and was saved only by using artificial respiration to get it under the anesthetic.

On account of the clinical similarity of this case to a case of tetanus which had come under our observation, and the similar condition in the spinal cord, we determined to give magnesium sulphate intraspinally. Following the advice of Blake<sup>2</sup> (8, 9), we injected 0.9 cc. of a 25 per cent solution in the fourth lumbar space, keeping the head well elevated. The anesthetic was removed. The child at once recovered, seemed lively, and in no danger from respiratory paralysis. Apparently there was some loss of motor control in both legs, but without loss of the knee jerk reflex. Morphine,  $\frac{1}{4}$  of a grain, was

<sup>2</sup> At that time we used the most easily available information as found in Keen's Surgery (Frazier, C. H., Tetanus, in Keen, W. W., Surgery, Philadelphia and London, 1907, i, 499). A more comprehensive article by Meltzer (19) since then advocates 0.5 cc. of a 25 per cent solution per 20 pounds in very young children.

given subcutaneously. The convulsions did not recur and the patient spent an undisturbed night, precautions being taken to keep the head elevated. Because of the slow excretion of strychnine in the urine, we thought that there might be a return of the condition the following day, but up to the time of discharge, December 5, 1915, there was no sign of even an increased motor irritability. A specimen of the urine was not obtained until December 4, at which time it contained no strychnine. Despite the large amount of arsenic also ingested, except for the vomiting and frequent evacuations of the bowels, the picture of acute arsenic poisoning was never presented, and later examination showed no evidence of chronic arsenic poisoning.

The action of strychnine (Kobert (1, 10), Tyrode (3), Cushny (4), Dixon (5), and Schmiedeberg (11)) consists in stimulation of the central nervous system, including the cord, medulla, and brain. Great reflex irritability results, with the characteristic rapid and active response to external stimuli. The action seems localized at some point between the entrance of the afferent fibers and the synapse about the motor cell. The easy response to stimuli from without seems best explained as the removing of some resistance "to the passage of impulses through some of the synapses in the spinal cord and thus extends the area on which an impulse acts, and also liberates it from the normal coördinating influences" (Cushny (4)). To control the action, then, it would seem reasonable to try to interpose an inhibiting influence at the point of the synapses.

In a series of papers Meltzer and Auer (12-18) discuss the toxicology, pharmacology, and therapeutic use of magnesium sulphate. They have demonstrated that the chief action of the drug is to inhibit and depress the central nervous system, simulating the action of cocaine when used locally. In spinal subdural injections its first action is to produce anesthesia, and later, with larger doses, paralysis. Thus it, too, may act in intraspinal injections, at much the same point as strychnine, though through a different mechanism and in an opposite direction.

In planning a series of experiments to test the efficacy of using magnesium sulphate to control strychnine convulsions, we found some confusion in the statistics recording the minimal lethal dose of strychnine. Githens and Meltzer (6) considered 0.0004 gm. per kilo of body weight fatal in dogs when administered intravenously. Hale<sup>3</sup> gave the same dosage from the results of his own experiments, also in dogs. Kobert (1) states that 0.0005 to 0.001 gm. per kilo is fatal for cats, dogs, sheep, and pigs.

<sup>3</sup> Personal communication.



## EXPERIMENTAL.

We first planned a series of experiments to determine the minimal lethal dose (Tables I and II). Healthy medium sized cats were used. Strychnine was administered subcutaneously in the flank.

TABLE I.

Experiment No.	Weight of animal.	Strychnine.		Result.	Remarks.
		Amount per kg.	Amount injected.		
	gm.	gm.	gm.		
1	2,970	0.0002	0.0006	Well.	No convulsions; increased reflex irritability.
2	2,700	0.00035	0.0010*	"	No convulsions; increased reflex irritability.
3	3,900	0.0004	0.0016	Death.	General convulsive seizures began in 20 min. Death with inhibitory respiration in 1 hr.
4	2,200	0.0004	0.0009	"	General convulsive seizures began in 40 min. Death in 2 hrs.
5	3,560	0.0005	0.00175	"	General convulsive seizures began in 20 min. Death in 1½ hrs.
6	3,260	0.0005	0.00162	"	General convulsive seizures began in 20 min. Death in 30 min.
7	2,700	0.00065	0.0018	"	General convulsive seizures began in 24 min. Death in 50 min.

\* In dealing with such small amounts of the drug the dosage given in some instances is approximately proportional to the animal's weight. not absolute.

TABLE II.

Experiment No.	Weight of animal.	Strychnine.		Result.	Remarks.
		Amount per kg.	Amount injected.		
	gm.	gm.	gm.		
8	1,405	0.0004	0.0006	Well.	Convulsion in 15 min. Ether to control it. Same procedure off and on for 1 hr. Then spastic, but no more convulsions.
9	2,970	0.0004	0.0012	Death.	Convulsions in 15 min. Controlled repeatedly by ether. Death in 1 hr. and 5 min.
10	3,150	0.0006	0.0018	"	Convulsions in 20 min. Controlled repeatedly by ether. Death in 1 hr.



These experiments convinced us that in cats the minimal lethal dose of strychnine was 0.0004 gm. per kilo. That we were able to control the convulsions entirely and prevent death by the use of ether (cone method) in Experiment 8, we attributed to the fact that the strychnine mixture as used in this experiment was a new one and not reliable in concentration. This preparation was discarded at once.

In the following experiments we went on the supposition that the same proportionate dose of the magnesium sulphate should be used intraspinaly, as in children; *i.e.*, 1 cc. of a 25 per cent watery solution per 25 pounds.<sup>4</sup> Some difficulty was experienced, at first, in performing lumbar puncture on cats, but it was found that the space between the sacrum and fifth lumbar vertebra is sufficiently large and easily located because of the surrounding bony prominences.

In the first series of experiments (Table III) we injected strychnine subcutaneously, waited for a convulsive attack, anesthetized the animal, and injected magnesium sulphate subdurally. In the second series (Table IV), we first anesthetized the animal and then simultaneously injected strychnine hypodermically and magnesium sulphate intraspinaly. We hoped by this method to show that we could abort the characteristic strychnine seizures. For anesthesia ether, by the cone method, was administered, except in one case in which chloroform was employed, and was used only long enough (often but 1 or 2 minutes), to enable us to complete our injections. The case, No. 12, in which we used chloroform died, probably because of the anesthetic, not the strychnine.

<sup>4</sup> The more recent advice of Meltzer (19) is 0.5 cc. per 20 pounds in very young children.

TABLE III.

Experiment No.	Weight of animal.	Strychnine.		Amount of magnesium sulphate.	Result.	Remarks.
		Amount per kg.	Amount injected.			
	gm.	gm.	gm.	cc.		
11	2,970	0.0004	0.0012	0.264	Well.	Convulsions in 20 min. After magnesium sulphate weak in hind legs, spastic in fore legs. Excitable but no more convulsions.
12	1,960	0.0004	0.0008	0.20	Death.	Severe convulsions in 30 min. Chloroform for 20 min. to control spasms. Then magnesium sulphate. Death before recovery from anesthetic.
13	3,060	0.0004	0.0012	0.375	"	For discussion see below. Magnesium sulphate cause of death.
14	3,900	0.0004	0.0016	0.352	Well.	Convulsions in 25 min. After magnesium sulphate paralyzed in hind legs. Quiet, no more convulsions.
15	3,450	0.0004	0.0014	0.308	"	Convulsions in 20 min. After magnesium sulphate weak in hind legs; slight spasm 1 hr. later; recovered from spasm.
16	3,260	0.0004	0.0014	0.286	"	Convulsions in 30 min. Weak in hind legs after magnesium sulphate. No more spasms.
17	3,235	0.0004	0.0013	0.286	"	Convulsions in 30 min. Weak in hind legs after magnesium sulphate. No more spasms.
18	2,970	0.0004	0.0012	0.264	"	Convulsions in 15 min. After magnesium sulphate no more spasms. Slight weakness in hind legs.
19	1,685	0.0004	0.0007	Total at 2 injections 0.308	"	Convulsions in 10 min. 15 min. after magnesium sulphate, repeated severe spasms in fore legs, none in hind legs. Dose of magnesium sulphate 0.154 cc. repeated. No more spasms. Playful with fore legs.
20	3,770	0.0004	0.0015	0.331	"	Convulsions in 25 min. No spasms after magnesium sulphate.

TABLE III—*Continued.*

Experiment No.	Weight of animal.	Strychnine.		Amount of magnesium sulphate.	Result.	Remarks.
		Amount per kg.	Amount injected.			
	gm.	gm.	gm.	cc.		
21	1,916	0.0004	0.0008	0.168	Well.	Convulsions in 30 min. No spasms after magnesium sulphate.
22	2,080	0.0004	0.0008	0.183	"	Convulsions in 50 min. No spasms after magnesium sulphate. Recovered. Died in 10 days of meningitis. The only case of infection.
23	3,235	0.0006	0.0019	0.280	Death.	Convulsions in 24 min. Flaccid in hind legs after magnesium sulphate. Repeated convulsions and death in 45 min.
24	2,880	0.0006	0.0017	0.260	"	Convulsions in 1 hr. and 25 min. After magnesium sulphate very excitable. Dead in cage next morning.

These experiments showed that we could save animals which had been given a lethal dose of strychnine, by the intraspinal injection of magnesium sulphate. The onset of the first strychnine convulsion though ushered in by a gradual increase in the irritability toward external stimuli, was usually very abrupt, and the spasms were so severe that frequently manual artificial respiration as a means of getting the animal under the effect of the anesthetic was undoubtedly the means of preventing death with the initial convulsion. The death in Experiment 12, was, we thought, due to the anesthetic. The death in Experiment 13 was due to magnesium sulphate poisoning. The effect of the drug as it diffused upwards in the spinal cord could be clearly seen. After recovery from the anesthetic the hind legs were noticed to be partially paralyzed, though frequent spasms seized the fore legs. Touching the hind legs called forth no reaction, while the slightest touch on the fur of a fore leg caused intense spasm of the upper half of the body. The animal was then laid on an inclined board with its head downwards. The effect of the magnesium sulphate moved steadily upwards, and areas on the abdomen and

chest which on previous light touch called forth spasm, no longer yielded the slightest response. Finally only the area from the neck upwards remained irritable. Soon afterwards the quality of the respiration became impaired and irregular. The head was then elevated and artificial respiration resorted to. Breathing was resumed and the animal lay in a semicomatose state for an hour longer, when respiration again failed and finally ceased.

The deaths in Experiments 23 and 24 were evidently caused by the excessive dose of strychnine. It does not seem improbable, however, that we could have saved the animals, by larger doses of magnesium sulphate, as we did in Experiment 19. In every case following the injection of the magnesium sulphate the same phenomena were observed: (1) flaccidity to almost total paralysis of the hind legs with failure of response to external stimuli; (2) continued increase of reflex irritability in the rest of the body, gradually diminishing and invariably progressing from the lower spinal region upwards. Few animals evinced any weakness in their hind legs 24 hours after the experiment.

In order to study further the power of magnesium sulphate in controlling the action of strychnine, we performed the following experiments, in which after anesthetization (necessary for the intraspinal injection), we injected strychnine and magnesium sulphate simultaneously (Table IV).

The results in these experiments seem to show that by using magnesium sulphate intraspinally early enough, the effects of the strychnine may be partially aborted. Possibly had we used larger doses of magnesium sulphate we should have been even more successful, but the death of the animal in Experiment 13 made us feel that we were near the high limits of the use of this drug.<sup>5</sup> The death of the animal in Experiment 27, though delayed, was probably due to strychnine, for we noticed no symptoms of the effects of the magnesium salts as late as 2 hours after the injection. Possibly in this case the full dose of the salt did not reach the subdural space. That the resultant convulsions in Experiments 28 and 29, despite the use of

<sup>5</sup> Meltzer (12) found that monkeys withstood 0.06 gm. per kilo intraspinally even when repeated two or three times at frequent intervals. Larger doses were fatal. In view of our experiments this seems a high dosage and suggests that strychnine may counteract the action of magnesium sulphate to some extent.

TABLE IV.

Experiment No.	Weight of animal.	Strychnine.		Amount of magnesium sulphate.	Result.	Remarks.
		Amount per kg.	Amount injected.			
	gm.	gm.	gm.	cc.		
25	3,150	0.0004	0.00126	0.268	Well.	In 30 min. weak in hind legs, but very irritable and susceptible to external stimuli. No convulsions.
26	2,690	0.0004	0.00107	0.240	"	Weak in hind legs and irritable. One general spasm in 35 min., then free from spasms.
27	2,500	0.0004	0.0010	0.220	Death.	In 35 min. one small convulsion. Weak in hind legs; irritable. Dead next morning.
28	2,880	0.0004	0.0012	0.260	Well.	In 20 min. severe spasm. Whiff of ether given. No more spasms.
29	3,170	0.0004	0.0012	0.276	"	In 30 min. severe spasm. Whiff of ether given. No more spasms; weak in hind legs.
30	2,455	0.0004	0.0010	0.220	"	Never any convulsions. Walked well. Slightly irritable.

magnesium sulphate, were so severe as to require a whiff of ether shows that the effect of the strychnine still dominated. Apparently there is a period about 20 minutes after subcutaneous injection when the toxic effect of strychnine is at its height. If at this time ether is given for a moment only, the magnesium salt will control further reactions.

## SUMMARY.

We feel that the results obtained in the human case and in our animal experiments justify the supposition that magnesium sulphate may be of use in controlling cases of poisoning by strychnine. It is a method easily available not only in large hospitals but in private practice, and requires no elaborate technique. The amount of magnesium sulphate to be used should follow the advice of Meltzer (19) in tetanus cases: intraspinally 1 cc. of a 25 per cent solution to each 20 pounds of body weight in adults, and one-half the dose in young children. Should this not control the convulsions a small amount of ether may be used. In order to hasten the excretion of



strychnine, 200 to 300 cc. of salt solution should be given intravenously.<sup>6</sup> In the event of the return of the convulsions, the intraspinal dose may be repeated, always taking precautions to keep the head elevated. Meltzer's apparatus for intrapharyngeal insufflation and a 2.5 per cent solution of calcium chloride should always be on hand in case of respiratory failure following an overdose of the magnesium salts (Meltzer (19) ).

We wish to express our indebtedness to Dr. S. J. Meltzer and Dr. J. Auer of The Rockefeller Institute for Medical Research for their interest and advice.

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<sup>6</sup> Githens and Meltzer (6) used Ringer's solution, but, of course, on account of the balancing action of calcium on magnesium salts (Meltzer (19) ), this solution could not be used. Ringer's solution less the calcium chloride content, however, might prove more efficacious than plain salt solution in the elimination of strychnine.



# STUDIES ON THE METABOLISM OF CELLS IN VITRO.

## I. THE TOXICITY OF $\alpha$ -AMINO-ACIDS FOR EMBRYONIC CHICKEN CELLS.

BY MONTROSE T. BURROWS, M.D., AND CLARENCE A. NEYMANN, M.D.

(*From the Pathological Laboratory and the Laboratory of Internal Medicine, Henry Phipps Psychiatric Clinic, Johns Hopkins University, Baltimore.*)

PLATE 13.

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A synthetic medium suitable for the growth of tissue cells outside of the animal organism has not been discovered up to the present time. Since the preparation of such a medium would lead directly to a better understanding of cellular metabolism, this problem has stood forth as one of the most important of those presented by the tissue culture method.

From earlier observations evidence had already been obtained which showed that the ingredients essential for the building of new cells and the liberation of energy in the cultures comes directly from the tissue fragments. The growth observed is a manifestation of a simple transfer of materials from the more central portions of the fragment to the cells at the periphery, or, in other words, the preying of one cell in a more suitable environment upon its neighboring cells in an unsuitable one. This is true in cultures where simple isotonic salt solutions have been used as the medium. That it is also true in the case of the plasma culture can be readily shown by repeatedly changing the medium or transplanting the cells to drops of fresh medium. All activity ceases after a few transplants, or when the cells within the fragment have become exhausted.

One of us,<sup>1</sup> in a recent study, has shown that tissue cells are not highly organized elements. Their various manifestations of life are the result of reactions between themselves and a specifically

<sup>1</sup> Read before Section VIII, Subsection E, Second Pan American Scientific Congress and the American Cancer Society, Washington, January 6, 1916.

organized environment. The form of the manifested change is determined by the peculiar organization and composition of the environment. Growth associated with mitotic cell division is seen only in cells which have become passively placed at the interspace between certain insoluble substances and the medium. These substances are liberated from the cells when they are removed from their normal habitat to an oxygen-containing plasma or salt solution. The substances are almost transparent, their refraction is not very different from that of the original medium, and they accumulate at the surface of the medium to form a membrane. They are liberated in large amounts from a tissue fragment rich in cells.

A cell brought in contact with the surface of this transparent substance adheres to it and flattens over its surface. Such a cell grows and divides by mitosis when oxygen is present and the waste products remain below a certain concentration.

A single isolated cell when placed in a drop of fresh plasma does not grow and divide. It may show movement. Connective tissue cells often assume spindle and irregular stellate shapes. These simple activities lead, however, apparently to the exhaustion of these cells. They come to rest after a short period and show evidences of deterioration through vacuolization and failure to stain sharply.

All cells that grow in these cultures grow at the expense of their neighbors. In the culture the cells at the border of the fragment grow; they have come in contact with a layer of the transparent substances mentioned above, which was liberated by the large number of cells deteriorating in the center of the fragment. These cells within the fragment about which the border cells are growing actively soon become reduced to mere shadows. This deterioration goes on to such an extent that they no longer stain.

With the increase in the concentration of the waste products the activity of the cells in the outer medium not only becomes less and less, but there is also a decrease in the liberation of substances from the fragment. Eventually the whole system comes to a state of complete inactivity or equilibrium. Such a system remains without further change often for a long period of time, sometimes as long as 6 months, at incubator temperature and in the presence of an ample supply of oxygen. If the cells and the fragment at any time during

this period are removed to fresh medium, normal activity is again observed, provided the fragments and the cells had not been transplanted frequently at a previous time, or become exhausted of this substance, or of substances which lead to its formation.

If cells are placed in a medium where the mechanical conditions are such that these substances cannot diffuse out from the fragment over a surface, or the cells are prevented from coming in contact with this surface, growth followed by mitotic division does not take place, but other forms of change result. By merely changing the mechanical organization of the culture or changing the differential surface tension of the cell it has been found possible to change its form, structure, and the manifested activity associated with it. Heart muscle cells when placed in a medium where they may be passively carried against the flat surface of these substances lose their myofibrillar structure and grow and divide by mitosis. They resemble in every way growing sarcoma cells. The same cells may again be caused to grow to form a syncytium, their nuclei dividing by a mitosis. Again they may be caused to round off and resemble large mononuclears, or to differentiate into myofibrils and *vice versa*. The contracting heart muscle cell is a cell which has become stretched through a serum cavity between the surface formed by the substances diffusing from the fragment and an elastic band of fibrin. One end is adherent to the surface and the other to the end of the band of fibrin. The cell is elongated and spindle in shape. Its elongated shape is maintained by the stress of the fibrin. The surface of the cell, which is unattached to the fibrin or the surface of the substances coming from the fragment, is sharply defined. It has a high surface tension. The points of active change in such a cell are evidently the points of contact.

The cells of higher animals are, therefore, strikingly different from many unicellular organisms, such as the paramecium, etc., which contain within themselves all the necessary organization for their growth. Growth and the various manifestations of life of the animal cell are differential surface tension phenomena which are under the control of the environment. Their manifestations of life are manifestations of "tissue rather than cellular reaction."<sup>1</sup>

The growth of the cells in the culture depends upon the presence

of certain specific substances which are liberated by their neighbors and a specifically organized environment. The growth itself is evidently a highly specific reaction. It is a reaction which one might readily assume would continue as long as these specific substances are liberated from the fragment, the proper mechanical organization is maintained about the cells, ample supply of oxygen is present, and the waste products of the reaction are removed.

The questions that confronted us primarily in a further study of these cultures are: What is the nature of these substances? How are they formed in the body under normal conditions? Can any part of the body live an independent existence when isolated from the other parts? Is the organization of the culture such that it will be able to form these substances from simple food bases? The unicellular organism is capable of breaking down complex substances and building its various component parts from the products liberated. Are these systems which have been isolated, tissue cultures, also capable of an independent existence? Is the failure to demonstrate any actual increase of protoplasm in the plasma culture indicative that these organizations are not able to live an independent existence, to build these substances from the food bases, or is it indicative merely of the lack of the proper food bases in the plasma which has been used as a medium?

The fact that plasma contains carbohydrates, proteins, and fats made it seem plausible to assume that these systems are unable to utilize them directly as food substances. That in the ordinary plasma cultures these food bases are insufficient for the building of new cells can be shown definitely not only by the failure of the cells to continue to grow after several transplants, but further by actual measurements. The sum of the masses of new cells formed in each transplant is always less than the original mass. One could readily consider in a well regulated series that it represents the original mass minus the energy of transfer.

#### EXPERIMENTAL.

In the present series of experiments we tried to determine whether the addition of any substance to the plasma would prolong the growth of the cells. The addition of certain carbohydrates and fats did not

affect the growth to any degree. During the course of further experiments we had the opportunity to try certain hydrolytic products of the protein of egg yolk. Since the results of these experiments have a certain interest in themselves, we decided to report them separately at this time.

### *Method.*

The tissues used for these experiments were heart muscle and pieces of the body wall of chick embryos and fetal chickens. The control medium for the experiments consisted of one part of fresh plasma containing a moderate amount of fat and one part of a 0.9 per cent sodium chloride solution. In the experiments the same proportions were used: one part of the isotonic solution of the substance to be tested was added to one part of the same plasma.

Fragments of heart muscle 1 mm. in diameter are placed in a layer of medium spread over the surface of a cover-glass. This layer has a thickness of 0.5 mm. A hollow ground slide is inverted over the drop, and the edge of the cover sealed to the slide with vaseline and paraffin. The cultures are placed in an incubator kept at a constant temperature of 39.4°C. They are examined under the microscope, which is kept in a chamber heated to the same temperature as that of the incubator.

After 3 days' incubation the growth of each is recorded and the fragment with the cells about it from each of the cultures is carefully transferred to a new drop of the medium, in which it has been previously grown. At the end of another 3 days the growth is again recorded and the fragments and cells again transplanted, and so on until no more activity is seen.

The growth in these cultures takes place as a rule along a single plane. A rough estimate of activity can be made by measuring the area covered by new cells about a fragment. There are always, however, a number of cultures in which the cells grow in several planes. On this account we have decided that a more accurate estimate can be given by the use of a relative term +, rather than by the direct numerical measurements. In the tables we have used this method of recording activity.

Plasma has been used in these experiments not only because it was of interest to test this medium more carefully, but also because



when it is used it is possible to obtain a constant and readily predicted cellular activity in the control cultures. This has not been found to be true for any of the simple liquid media. The proper mechanical conditions in the latter cultures are difficult of manipulation.

In the first series of experiments we prepared peptones from the yolk of eggs; that is, they were prepared from the specific food proteins of the embryonic chicken cells. These peptones were obtained by the use of the general method described by Abderhalden<sup>2</sup> and slightly modified by us as follows: 240 gm. of coagulated egg yolk are dried and pulverized, and treated with 1,000 gm. of 70 per cent (by weight) of pure sulphuric acid. The coagulated yolk does not dissolve as readily as one might wish, so the mixture is shaken vigorously for an hour. A thick scum forms on top, the rest of the material going into solution. This scum is removed. It proves to be fat. The brownish purple solution is next allowed to stand at room temperature for 3 days. It is frequently shaken during this time and then transferred into a 20 liter mixing jar, the latter being packed in a freezing mixture where it is cooled thoroughly. 10 liters of water are then added very slowly, while stirring, keeping the temperature below 20°C. After this, enough barium hydroxide in crystalline form is added to give the solution a neutral reaction towards litmus. This process requires great care. It is best to calculate the exact quantity of barium hydroxide needed, to add nearly this quantity, and then proceed gradually. The solution must be stirred constantly in order to keep the temperature below 20°C. at all times. The voluminous precipitate of barium sulphate is best separated from the digested solution by means of a centrifuge. This solution is next treated alternately with 0.1 N barium hydroxide and 0.1 N sulphuric acid until the addition of an excess of either to a filtered or centrifuged sample does not give a further precipitate of barium sulphate. Of course, an addition of barium hydroxide to a peptone solution will sometimes give a precipitate of barium peptonates, but these are soluble in dilute nitric acid, while barium hydroxide is not. Thus an absolutely neutral solution is prepared. This solution of over 10 liters in quantity is next concentrated under

<sup>2</sup> Abderhalden, E., *Defensive Ferments of the Animal Organism*, New York, 1914, 204.



reduced pressure until about 100 cc. of a syrupy, light yellow liquid results. This is treated with about 2 liters of hot methyl alcohol and filtered into 10 liters of chilled ethyl alcohol. A white precipitate results, and is quickly filtered off by the aid of a suction pump and dried in a desiccator. The color of the resultant powder is light yellow.

These peptones gave all the true peptone reactions. They were absolutely soluble in a saturated solution of ammonium sulphate and remained unchanged as regards color on boiling with a solution of sodium hydroxide to which a drop of lead acetate solution had been added. They were dialyzable through a dialysis tube, impervious to albumin, and the dialysate gave a typical blue ninhydrin reaction. Finally, they gave a reddish biuret reaction, and their microscopic appearance was similar to that of commercial silk peptone.

A large amount of this peptone, namely, 2.5 per cent, can be dissolved in 0.85 per cent sodium chloride solution, without making the solution hypertonic, and thereby killing the cells. This solution is mixed half and half with plasma. The growth, as is shown in Table I, Cultures 1, 2, 3, 4, and 5, continued regularly up to the sixth transplant. After this the various fragments became exhausted. The control cultures, 6, 7, 8, 9, and 10, behaved in exactly the same manner. From this we conclude that peptones do not in any way enter into this growth reaction. They did not prolong it, and no change of any kind was noted by their addition.

We next turned our attention to the study of  $\alpha$ -amino-acids. The complete hydrolysis of egg yolk promised a yield of  $\alpha$ -amino-acids in the same proportions as they actually occur in the protein molecule, the only  $\alpha$ -amino-acid which does not give a yield on acid hydrolysis being tryptophane. This seemed to be the easiest and most direct way of procedure. Consequently a digestion mixture was prepared by dissolving dried egg yolk from which the fats had been extracted with ether by means of a Soxhlet apparatus, in a 70 per cent (by weight) solution of sulphuric acid. After the protein had gone into solution, enough water was added to make this a 25 per cent solution of sulphuric acid. The entire mixture was then heated in a flask with a reflux condenser until a biuret reaction was no longer obtainable. This acid solution was next neutralized with

TABLE I.

*Tissue: Fragments of the heart muscle of a 12 day old chicken embryo.*

*Medium: Isotonic solutions of peptone, 2.5 per cent, in sodium chloride solution, 0.85 per cent, 1 part.*

*Plasma, 1 part.*

*Control Medium: Sodium chloride solution, 0.9 per cent, 1 part.*

*Plasma, 1 part.*

Solution used.	Culture No.	Growth of transplants.						
		1st.	2nd.	3rd.	4th.	5th.	6th.	7th.
Peptone.	1	++++	++++	++	+	0	0	0
	2	+++	++++	+++	++	Few cells.	0	0
	3	+++	+++++	+++	+++	+	Few cells.	0
	4	++++	++++	++++	+++	++	Few cells.	0
	5	++++	++++++	++++++	++++++	0	0	0
Control.	6	+++	+++++	—*	—	—	—	—
	7	+++++	++++++	++++	++	0	0	0
	8	+++	++++	++	++++++	++	Few cells.	0
	9	+++++	++++++	++++++	++++	0	0	0
	10	+++	++++	++	++	Few cells.	0	0

\* In the tables the dashes indicate that the culture was lost through breaking of the glass slide, infection, or other causes.

barium hydroxide in exactly the same manner as stated above for the preparation of peptones. After this the solution was concentrated under reduced pressure at 40°C. until evaporation to dryness gave a 3 per cent residue, which we believe consists for the most part of  $\alpha$ -amino-acids. Plasma was diluted with this mixture. To our surprise all the cells were dead in 48 hours, while the control cultures in plasma alone grew and kept on growing as usual when they were transplanted. We did not consider this peculiar result due to the possible toxicity of the  $\alpha$ -amino-acids as such, but thought that traces of sulphuric acid or barium hydroxide caused the death of the cells. In order to make sure that this was the case, a part of the digestion mixture was further concentrated to one-fifth of its

volume, divided in two portions, and each portion tested with an excess of 0.1 N sulphuric acid and 0.1 N barium hydroxide respectively. No precipitation occurred.

Discarding the idea that a trace of sulphuric acid or barium hydroxide could be the cause for failure, we considered hypertonicity as a possibility. A simple way of testing whether a solution is isotonic or not towards chicken cells is by the use of chicken erythrocytes. The red blood corpuscles are nucleated and consequently have a bulge in the middle of the disc. This bulge is especially conspicuous when the corpuscle is seen on end. If a solution is hypotonic, the surrounding disc will swell and this bulge will become less marked. If, on the other hand, the solution is hypertonic, one sees characteristic shrivelling around the edge of the disc. It is, of course, understood that extremely hypotonic or hypertonic solutions will cause a dissolution of the corpuscles, a dissemination of the hemoglobin, and give a characteristic laked appearance.

Making up various dilutions of the digestion mixture, we transferred 1 cc. of the dilutions to suitable test-tubes and added 0.25 cc. of a 5 per cent suspension of washed chicken erythrocytes in 0.9 per cent sodium chloride solution. We then incubated it for 4 hours and compared it with the form of our control diluted with 0.9 per cent sodium chloride solution and treated in exactly the same manner. Using the dilution which showed neither shrivelling nor bulging, we prepared an isotonic digestion mixture.

Renewed experiments with the digestion mixture gave practically the same results as before. A repetition of the experiment with a new digestion again resulted in the death of the cultures. A portion of the last digestion mixture was treated with hydrochloric acid gas to the point of saturation and allowed to stand in the ice box for several days. We obtained a voluminous precipitate of glutamic acid hydrochloride, showing its typical crystalline form. This gave the final proof that  $\alpha$ -amino-acids were present. Even now we did not attribute our results to a toxic influence of the acids in themselves but rather to the influence of the brown coloring matter in the solution, said to consist of caramelized carbohydrates, humin substances, etc. We therefore tried pure  $\alpha$ -amino-acids.

We used ten of the acids in our first experiment; namely, glycoll,

alanine, cystine, valine, leucine, phenylalanine, tyrosine, tryptophane, oxyproline, and asparagine.<sup>3</sup> Some of the substances were synthetic (Merck and Kahlbaum preparations), while others were obtained by the hydrolysis of proteins.<sup>4</sup>

To be sure of the substances which we used, the total nitrogen of each of the  $\alpha$ -amino-acids was determined, their melting points were ascertained, and their crystalline forms observed. All these tests proved that we were dealing with chemical substances of the right molecular constitution containing no demonstrable impurities.

Hot saturated solutions of these acids were prepared. The solutions were cooled, and each solution was tested with chicken erythrocytes for isotonicity by the methods described above. The more insoluble substances, such as cystine, were primarily dissolved in isotonic salt solution, as we could hope to obtain isotonicity in no other way. All these solutions were diluted half and half with plasma and used as culture media.

Tables II, III, and IV give a survey of the experiments. In every case the addition of a solution of  $\alpha$ -amino-acids to the plasma results in the ultimate death of the growing cells, while the controls keep on with their regular development. The discrepancy between the time required to kill the cultures described in Tables II and III, on the one hand, and in Table IV, on the other, the cultures of the latter series dying more quickly, is explained by the age of the embryo used. The cells of the younger embryo grow much faster and are less resistant to toxic influences than are those of an older embryo, as is known from numerous reported experiments.

<sup>3</sup> We used asparagine in spite of the fact that it has not been found as a constituent of hydrolyzed animal proteins. The effect as shown in the tables is exactly the same as in the case of the other  $\alpha$ -amino-acids. Fig. 2 is a fair representative of the appearance of any one of the cultures in which  $\alpha$ -amino-acids had been added to the medium. This culture was selected for photographing because it was prepared practically at the same time as the controls. The tissue that was used was most similar and had suffered practically the same exposure as that used for the controls.

<sup>4</sup> We wish to thank Dr. P. A. Levene, of The Rockefeller Institute for Medical Research, Dr. D. W. Wilson, of the Physiological Chemical Laboratory of Johns Hopkins University, and Dr. R. F. Ruttan, of McGill University, for supplying us with some of the acids more difficult to manufacture.

TABLE II.

*Tissue: Fragments of the heart muscle of a 12 day old chicken embryo.*

*Medium: Isotonic solution of the  $\alpha$ -amino-acid, 1 part.*

*Plasma, 1 part.*

*Control Medium: Sodium chloride solution, 0.9 per cent, 1 part.*

*Plasma, 1 part.*

Solutions used.	Culture No.	Growth of transplants.	
		1st.	2nd.
Glycocoll, 35 per cent solution, * 1 part, and water, 19 parts.	1	+++	Few cells.
	2	+++	" "
	3	+++	" "
	4	+++	0
	5	+++	—
Alanine, 11 per cent solution, 1 part, and water, 13 parts.	6	++	+
	7	+++	Few cells.
	8	+++	+
	9	+++	0
	10	+++	Few cells.
Valine, 8 per cent, 1 part, and water, 4 parts.	11	+++	Few cells.
	12	++	0
	13	+++	Few cells.
	14	+++	0
	15	++	—
Leucine, 1.66 per cent, 1 part, and water, 2 parts.	16	++++++	0
	17	+++++	0
	18	+++	0
	19	+++++	0
	20	++++++	0
Cystine, 0.1 per cent solution, not diluted.	21	++	0
	22	+++	Few cells.
	23	++	+
	24	+++	+
	25	+++	0
Control.	26	+++	—
	27	++++++	+++++
	28	++++++	—
	29	+++++	+++++
	30	+++++	+++++

\* All percentages given are approximate.



TABLE III.

*Tissue: Fragments of the heart muscle from an 11 day old chicken embryo.*

*Medium: Isotonic solution of the  $\alpha$ -amino-acid, 1 part.*

*Plasma, 1 part.*

*Control: Sodium chloride solution, 0.9 per cent, 1 part.*

*Plasma, 1 part.*

Solutions used.	Culture No.	Growth of transplants.			
		1st.	2nd.	3rd.	4th.
Valine, saturated solution, cold, 1 part, and water, 3 parts.	1	+	Few cells.	0	
	2	+	0	0	
	3	+	0	0	
	4	Few cells.	0	0	
Glycocoll (Merck), saturated solution, cold, 1 part, and water, 24 parts.	5	++	++	0	
	6	+++	+++	0	
	7	++	+++	0	
	8	+	+++*	0	
Asparagine, saturated solution, cold, 1 part, and water, 3 parts.	9	++	+	0	
	10	+	+	0	
	11	+	+	0	
	12	+++	++	0	
Cystine, saturated solution, cold, 3 parts, and water, 2 parts.	13	+++++	+++	Few cells.	
	14	++++	++++	" "	+ Cells rounded off.*
	15	++++	++++	—	
	16	++++	—	—	
Controls.	33	+++++	+++++	+	+
	34	++	+++++	+	+
	35	++	+++++	+	+
	36	+++++	+++++	+	+

\* All the pieces are contracting actively.

In Table V, we tried more dilute solutions, and, as this table indicates, the cells tend to resist this medium for a longer time. However, none of these cultures grew as long as the controls, which shows that even in very low dilutions the  $\alpha$ -amino-acids are toxic. It is of interest in this series to note evidences of stimulation. This stimulation as the table indicates is not a stimulation of growth, but a stimulation of the contraction of the heart muscle fragments.



TABLE IV.

*Tissue: Fragments of the body wall of a 5 day old chicken embryo.*

*Medium: Isotonic solution of the  $\alpha$ -amino-acid, 1 part.*

*Plasma, 1 part.*

*Control Medium: Sodium chloride solution, 0.9 per cent, 1 part.*

*Plasma, 1 part.*

Solutions used.	Culture No.	Growth after 48 hrs.	Growth after 72 hrs.
Glycocoll, 35 per cent solution, 1 part, and water, 19 parts.	1	+	The cells have all rounded off and show distortion.
	2	Few cells.	
	3	" "	
Alanine, 11 per cent solution, 1 part, and water, 13 parts.	4	+	The cells have all rounded off and show distortion.
	5	Few cells.	
	6	" "	
Cystine, 0.1 per cent solution, not diluted.	7	+	The cells have all rounded off and show distortion.
	8	+	
	9	+	
Valine, 8 per cent, 1 part, and water, 4 parts.	10	+	+++ The cells have all rounded off.
	11	++	
	12	++	
Leucine, 1.66 per cent, 1 part, and water, 2 parts.	13	++	++ The cells have all rounded off.
	14	++	
	15	+++	
Phenylalanine, 2 per cent, slightly hypotonic, not diluted.	16	++	The cells have all rounded off and show distortion.
	17	—	
	18	+	
Tyrosine, 0.285 per cent in 0.85 per cent sodium chloride, 2 parts, and water, 1 part.	19	+	+ The cells have all rounded off and show distortion.
	20	++	
	21	++	
Tryptophane, 1.66 per cent in 0.85 per cent sodium chloride, 1 part, and water, 2 parts.	22	++	The cells have all rounded off and show distortion.
	23	+++	
	24	++	
Oxyproline, 10 per cent, 1 part, and water, 2 parts.	25	+	The cells have all rounded off and show distortion.
	26	++	
	27	++	
Asparagine, 15 per cent, 1 part, and water, 1 part.	28	Few cells.	The cells have all rounded off and show distortion.
	29	" "	
	30	" "	
Control.	31	+++	+++++
	32	+++	
	33	+++	

All the fragments contracted actively with the exception of the controls. This contraction continued to the complete exhaustion of the fragments.

TABLE V.

*Tissue: Fragments of the heart muscle from an 11 day old chicken embryo.*

*Medium: Isotonic solution of the  $\alpha$ -amino-acid, 1 part.*

*Sodium chloride solution, 0.9 per cent, 1 part.*

*Plasma, 2 parts.*

*Control: Sodium chloride solution, 0.9 per cent, 1 part.*

*Plasma, 1 part.*

Solutions used.	Culture No.	Growth of transplants.			
		1st.	2nd.	3rd.	4th.
Valine, saturated solution, cold, 1 part, and water, 3 parts.	17	++++	—	—	—
	18	++++	+ Few cells.	0	0
	19	++	—	—	—
	20	+++	++++	+++*	+++* The cells have all rounded off.
Glycocoll (Merck), saturated solution, cold, 1 part, and water, 24 parts.	21	++++	+++++*	+++*	+++++* The cells have all rounded off.
	22	+++++	+++++	+++*	0
	23	++++	++++	—	—
	24	—	—	—	—
Asparagine, saturated solution, cold, 1 part, and water, 3 parts.	25	++++	+++++*	—	—
	26	++++	+++++*	++++*	++++*
	27	—	—	—	—
	28	—	—	—	—
Cystine, saturated solution, cold, 3 parts, and water, 2 parts.	29	+++*	+++++*	+	0
	30	+++++*	+++++	+++*	0
	31	+++++*	+++++*	++++*	0
	32	+++++*	+++++*	+++*	0
Controls.	33	+++++	+++++	+++	+++++
	34	++	+++++	++++	+++++
	35	++	+++++	++++	++++
	36	+++++	+++++	+++++	+++

\* All the pieces are contracting actively.

## DISCUSSION AND SUMMARY.

Summing up these results, we found that all the ten  $\alpha$ -amino-acids used inhibited the growth of the cells and finally killed the cultures. This inhibition is preceded by a short period of activity. The typical effect on the cells is shown in Figs. 1 and 2. The first (Fig. 1) is a control culture showing the usual growth of cells and their typical spindle shape form. The second (Fig. 2) is a culture in plasma plus asparagine,<sup>3</sup> showing the cells rounded off and beginning to undergo dissolution.

We do not wish to draw too extensive conclusions from these experiments, but we believe that the toxicity of  $\alpha$ -amino-acids towards growing cells has been shown beyond a reasonable doubt; while we have found that compounds of higher molecular weight, namely, the peptones of egg yolk and proteins, are non-toxic. This toxicity depends upon the concentration and the time that the cells are exposed to their action. As these factors are reduced, the toxicity is decreased. In this respect, these substances are similar to all cell poisons.

Applying these results to the work done on the intravenous injection of digestion mixtures, we believe that we have found a reason for the death of the experimental animals when the hydrolyzed proteins were injected too rapidly. Buglia<sup>5</sup> found that large amounts of  $\alpha$ -amino-acids could be injected into the circulation without causing deep-seated changes in the renal and intestinal functions, provided they were injected slowly enough; in fact, that enough of these mixtures could be injected in this way to cover the nitrogen consumption of the body. This injection, however, was always accompanied by an  $\alpha$ -amino excretion through the urine and an increase of the peristalsis of the intestine, with resultant liquid stools. As is well known, a sudden great concentration of these substances in the blood of an animal causes death. These results agree with our findings.

Folin and Denis<sup>6</sup> demonstrated the fact that  $\alpha$ -amino-acids probably pass into the circulation through the intestines. Van Slyke

<sup>5</sup> Buglia, G., *Z. Biol.*, 1912, xviii, 162.

<sup>6</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 141.

and Meyer,<sup>7</sup> by means of Van Slyke's<sup>8</sup> nitrogen method, have practically proven this, and Abel, Rowntree, and Turner,<sup>9</sup> and Abderhalden<sup>10</sup> have lately succeeded in obtaining  $\alpha$ -amino-acids in crystalline form from the blood. Van Slyke and Meyer<sup>11</sup> have shown that the tissues take up  $\alpha$ -amino-acids to a certain point, but that after this the limit of saturation is reached. This is not so in the liver, which continually desaturates itself by metabolizing the  $\alpha$ -amino-acids that it has absorbed, and consequently maintains indefinitely its power of removing them from the circulation, as long as they enter it no faster than the liver can metabolize them. Marshall and Rowntree<sup>12</sup> have shown that there is an increase of the  $\alpha$ -amino-acid concentration in the blood after injuries to the liver, which have caused deep-seated anatomical changes. Our experiments prove that tissue cells in general are unable to live in the presence of any great concentration of these acids.

At the present time we do not feel able to give an explanation of the significance of this evident toxicity. However, the fact in itself seems to indicate that we should expect stimulation from a certain increase of the  $\alpha$ -amino-acid concentration in the body, or the concentration of any one of the acids, while a greater increase would lead to marked disturbances of the metabolism.

#### EXPLANATION OF PLATE 13.

FIG. 1. The living cells which had grown from a fragment of the body wall of a 5 day old chick embryo into a medium consisting of 1 part of plasma and 1 part of isotonic salt solution, 0.9 per cent.

FIG. 2. The degenerated cells which had grown from a fragment of the body wall of the embryo used in the culture in Fig. 1 into a medium consisting of 1 part of plasma and 1 part of an isotonic solution of asparagine.

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<sup>7</sup> Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

<sup>8</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185; 1912, xii, 275.

<sup>9</sup> Abel, J. J., Rowntree, L. G., and Turner, B. B., *J. Pharm. and Exp. Therap.*, 1913-14, v, 275, 611.

<sup>10</sup> Abderhalden, *Z. physiol. Chem.*, 1913, lxxxviii, 478.

<sup>11</sup> Van Slyke and Meyer, *J. Biol. Chem.*, 1913-14, xvi, 213.

<sup>12</sup> Marshall, E. K., and Rowntree, L. G., *J. Exp. Med.*, 1915, xxii, 333.

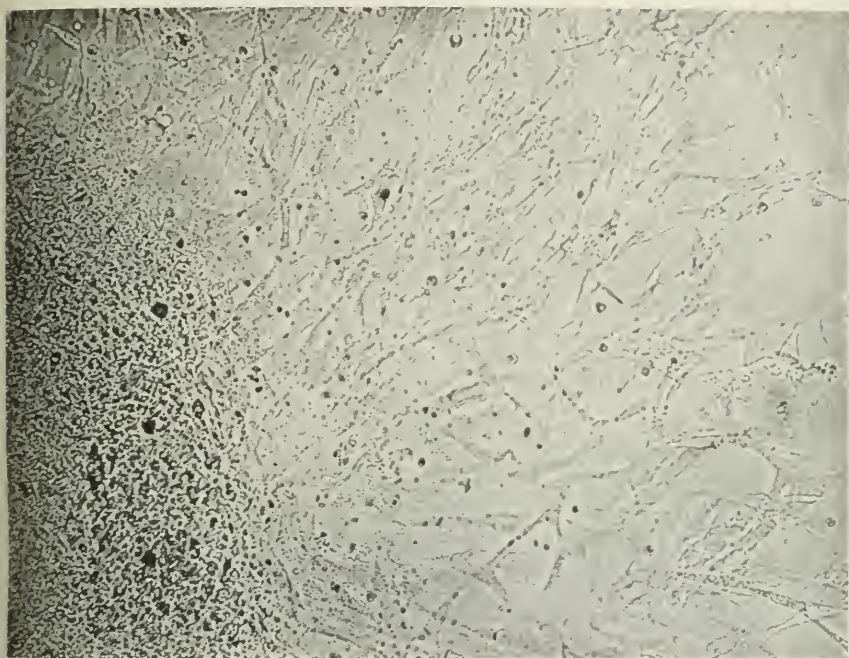


FIG. 1.

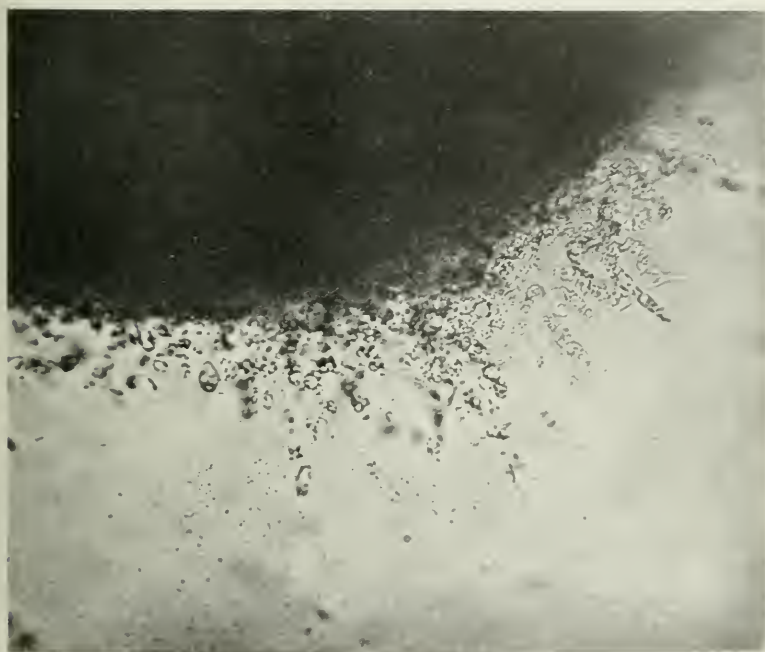


FIG. 2.

(Burrows and Neymann: Metabolism of Cells *in Vitro*. L.)





# THE PRODUCTION OF ARTERIOSCLEROSIS AND GLOMERULONEPHRITIS IN THE RABBIT BY INTRAVENOUS INJECTIONS OF DIPHTHERIA TOXIN.

By C. H. BAILEY, M.D.

(From the Pathological Laboratory of the Medical School of Leland Stanford Junior University, San Francisco.)

PLATES 14 TO 19.

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Attention has frequently been called to the presence of vascular lesions in man, especially fatty streaks in the aorta, following infectious diseases, such as diphtheria, scarlet fever, and typhoid. This association has naturally led to an assumption by many of an etiological relation between infectious diseases and arteriosclerosis and to frequent attempts to produce vascular lesions in animals by the injection of bacteria or their toxins. Since the limits of this paper do not permit of an extensive review of this subject brief mention only will be made of the more important work and that which seems to bear especially on the experiments here reported. Critical reviews of the subject have been made by Klotz (1, 2), Saltykow (3), Rickett (4), and Frothingham (5).

Gilbert and Lion (6) in 1889 traumatized the intima of the rabbit's aorta by passing a stylet down the carotid artery and afterwards injected cultures of *B. typhosus*. They thus obtained vegetative lesions. They also injected into two rabbits, without previous traumatization of the aorta, cultures of a bacillus obtained from a case of endocarditis. They produced plaques in the ascending portion and arch of the aorta, affecting mainly the media and consisting of fibrous tissue with some calcification.

Crocq (7) reported that traumatism of the aorta alone, by a method similar to that of Gilbert and Lion, produced no lesions; also that intravenous and subcutaneous injection of typhoid bacilli, colon bacilli, streptococci, and diphtheria bacilli, without previous traumatization, produced no lesions, but that when these bacteria were injected after previous injury to the aorta vegetations and plaques of endocarditis were produced. He therefore concludes that two factors are necessary, infection and a *locus minoris resistentiæ*.

Thérèse (8) and Pernice (9) injected *Staphylococcus aureus*, streptococcus, *B. typhosus*, *B. diphtheria*, etc., into rabbits and guinea pigs without previous injury to the aorta. They obtained microscopical lesions only which consisted of round cell infiltration and later fibrosis about the vasa vasorum, affecting mainly the adventitia, but in the more severe cases extending to the other coats.

Boinet and Romary (10) also tried trauma combined with infection. They used various bacteria, including diphtheria bacilli and also filtrates of cultures. Raised yellow patches of small extent were produced in the aorta which sometimes did not correspond to the traumatized areas. Microscopically these consisted of nodules of infiltration about the vasa vasorum with overlying plaques of endarteritis.

Saltykow (3) by repeated injections of staphylococcus into rabbits obtained small plaques of fibrous thickening and fatty degeneration of the intima of the aorta and some of its main branches, and sometimes cellular connective tissue nodules with calcification in the upper media.

Klotz (1) reports the production, by the injection of *B. typhosus* and streptococcus, of wart-like thickenings involving the intima and inner part of the media of the first part of the pulmonary artery and ascending limb of the aorta. He also mentions, without giving details of his experiments, the production of a vascular lesion in the rabbit with diphtheria toxin. On this subject he says: "The repeated inoculations of diphtheria toxin into rabbits gave surprising results. Here, instead of meeting with proliferative changes, such as the *B. typhosus* and streptococcus produce in the aorta, there were only lesions of a degenerative character. The degenerations were isolated to the first part of the aorta, and were identical with those produced in the adrenalin series. The thinning of the arterial wall, with calcification and aneurysmal dilatations, were all present, and the microscopical examination showed the lesions to be confined to the media. No proliferative or inflammatory changes were present in the intima, nor was there any change about the vasa vasorum."

Frothingham (11) remarks the fact that none of the vascular lesions which have been experimentally produced with bacteria or their toxins in animals are of so severe a type as those seen in man, and with the object of studying the effect of diphtheria toxin on the arteries he injected nine rabbits subcutaneously or intravenously. Some of these showed partial necrosis of tufts of the glomeruli with fibrin formation and in cases also there was necrosis of the walls of some of the larger vessels in the kidney with deposition of fibrin. Capillary lesions were also present in the adrenals. In no case were lesions of any of the larger arteries observed. Only two, however, of the nine rabbits survived over 3 days. One of these had five injections at intervals of 11 to 18 days and was killed at the end of about 10 weeks. The other had four injections at similar intervals and was killed in about 7 weeks. Two guinea pigs were also studied which survived an injection of toxin and antitoxin for about a month. No vascular lesions were present. The author concludes that though the glomeruli and smaller vessels in the rabbit's kidney are susceptible to diphtheria toxin, and also the capillaries

in the adrenal, "the vessels of these animals, however, are not on the whole sensitive enough to this toxin to make it seem hopeful to secure permanent lesions by the administration of sublethal doses."

The experiments here reported were undertaken in the hope of producing an arteriosclerosis of the larger vessels of the rabbit with diphtheria toxin. Lesions of the small vessels in certain organs have been described by various authors, as by Babes (12), Welch and Flexner (13, 14), and Frothingham (11) in the kidney, and by Mollard and Regaud (15) in the heart. The attempts of the earlier workers, in some cases successful, as quoted above, to produce vascular lesions by trauma and infection combined leave one in considerable doubt as to the relative importance of these two factors in the production of the lesion. The lesions obtained by Thérèse (8) and by Pernice (9) with bacteria or their toxins were slight and hardly comparable in character, severity, or extent with those in man which are suspected by some observers of being due to a preceding infection.

#### EXPERIMENTAL.

With the exception of the observation of Klotz (1), with which, however, the writer was unacquainted until these experiments were practically completed, the previous work seemed to afford little hope of producing arteriosclerosis with diphtheria toxin alone. An incentive to attempt to produce vascular lesions by the combined action of increased blood pressure and a toxin was furnished by a theoretical discussion of the etiology of arteriosclerosis by Aschoff (16). A number of rabbits were consequently injected with diphtheria toxin alone, a second series with diphtheria toxin and pituitrin, and a number, as controls, with pituitrin alone. The pituitrin was obtained from Parke, Davis and Company. It was given always intravenously. For the rabbits in Table II, it was usually mixed with the diluted diphtheria toxin, but occasionally was given undiluted a few minutes after the intravenous injection of the toxin; to the rabbits in Table III it was given undiluted. Pituitrin was selected because it was believed that of itself it would produce no vascular degeneration. This view was confirmed by the control rabbits Nos. 33, 34, and 35. Adrenalin was unsuitable on account of the medial degeneration produced by it in the aorta, as first pointed out by Josué (17), and since confirmed by many others.

All rabbits were injected with a single sample of diphtheria toxin obtained from the Cutter Laboratories, dilutions of which, freshly made each day before injection with sterile 0.85 per cent salt solu-

tion, were of such strength that the total amount of the injection was between 0.25 and 1 cc. The actual doses of undiluted toxin are given in the tables. The 0.4 per cent tricresol, added to the toxin by the manufacturers as a preservative, was disregarded under the assumption that the exceedingly small amount injected was without demonstrable morphological effect. All injections were made into the ear vein. The doses of toxin, of toxin and pituitrin, and of pituitrin alone are given in Tables I, II, and III. All the rabbits died from the effects of the treatment, except Nos. 33 and 34 which were killed at the expiration of the intervals shown in the tables.

All the animals were carefully autopsied with special attention to gross changes in the vessels. Frozen sections of various vessels and organs were stained with Sudan III and hematoxylin and with Van Gieson's stain and hematoxylin. Paraffin sections were stained with Van Gieson's stain and hematoxylin and with eosin and hematoxylin, and those of the vessels also with von Kossa's stain for calcium.

TABLE I.

*Rabbits Injected with Diphtheria Toxin.*

Rabbit No.	Weight.	Dosage of diphtheria toxin.	No. of doses.	Total dosage.	Duration of experiment.	Gross arterio-sclerosis.	Nephritis.
	gm.	cc.		cc.	days		
1	2,340	0.02	1	0.02	1½	Absent.	Fatty degeneration.
2	2,520	0.015	2	0.03	6	"	Acute glomerulo.
3	2,470	0.006-0.007	4	0.025	4	"	Slight acute glomerulo.
4	2,985	0.004-0.005	4	0.017	6½	"	Moderate acute glomerulo.
5	2,240	0.003-0.004	3	0.013	9	"	Acute glomerulo.
6	2,245	0.002-0.003	9	0.019	28	Present.	Subacute glomerulo.
7	1,640	0.002	5	0.01	5	Absent.	Moderate acute glomerulo.
8	1,655	0.002	5	0.01	5	"	" " "
9	2,260	0.002	4	0.008	8	"	Acute glomerulo.
10	2,650	0.002	4	0.008	8	Present.	Beginning subacute glomerulo.
11	1,870	0.0015-0.0025	11	0.019	21	"	Moderate " "
12	1,670	0.0015	4	0.006	10	Absent.	Beginning " "
13	1,880	0.001-0.002	17	0.0235	24	"	Moderate " "
14	2,010	0.001-0.0015	12	0.0135	20	Present.	Subacute glomerulo.
15	1,900	0.0005-0.001	17	0.0125	26	Absent.	Slight subacute glomerulo.

TABLE II.

*Rabbits Injected with Diphtheria Toxin and Pituitrin.*

Rabbit No.	Weight.	Dosage of diphtheria toxin.	No. of doses of diphtheria toxin.	Total dosage of diphtheria toxin.	Pituitrin.	Duration of experiment.	Gross arterio-sclerosis.	Nephritis.
	gm.	cc.		cc.		days		
16	2,010	0.03	1	0.03	2 doses of 0.4 cc.	1½	Absent.	Fatty degeneration.
17	3,200	0.02	1	0.02	2 " " 0.7 "	7	Present.	Acute glomerulo.
18	3,400	0.02	1	0.02	1 dose " 0.7 "	2½	Absent.	" "
19	3,450	0.02	1	0.02	1 " " 0.7 "	4	"	Slight acute glomerulo.
20	2,640	0.02	1	0.02	1 " " 0.7 "	2½	"	Slight glomerulo.
21	2,900	0.015	1	0.015	3 doses " 0.7 "	2½	"	Acute "
22	2,860	0.01	2	0.02	4 " " 0.7 "	3	"	" "
23	2,545	0.006-0.007	4	0.025	3 " " 0.7 "	5	"	" "
24	2,600	0.005	2	0.01	5 " " 0.7 "	5	"	Moderate acute glomerulo.
25	2,030	0.004-0.005	3	0.013	3 " " 0.7 "	5	"	Acute glomerulo.
26	3,360	0.003-0.004	4	0.013	4 " " 0.7 "	10	"	" "
27	2,645	0.002-0.003	4	0.009	4 " " 0.7 "	11	Present.	" "
28	1,465	0.002	5	0.01	5 " " 0.7 "	4	Absent.	" "
29	1,575	0.002	5	0.01	5 " " 0.7 "	9	"	" "
30	2,575	0.002	6	0.012	6 " " 0.75 "	14	Present.	Beginning sub-acute glomerulo.
31	2,240	0.0015-0.002	10	0.017	10 " " 0.75 "	20	"	Beginning sub-acute glomerulo.
32	1,615	0.0015	5	0.0075	5 " " 0.7 "	9	Absent.	Acute glomerulo.

TABLE III.

*Rabbits Injected with Pituitrin.*

Rabbit No.	Weight.	Pituitrin.	Duration of experiment.	Arteriosclerosis.	Nephritis.
	gm.		days		
33	2,260	4 doses of 0.5 cc.	4	None.	None.
34	3,110	23 doses of 0.5 to 1.0 cc.	23	"	"
35		2 doses of 0.8 cc.	3	"	"



In Tables I and II are also indicated the rabbits in which gross vascular lesions were found. A brief record of the vascular findings in these eight rabbits follows.

*Rabbit 6.*—The aorta is irregularly dilated especially in the thoracic portion. To the touch it is definitely stiffened. The large abdominal vessels, subclavians, and carotids are stiffened and the carotids show irregular dilatations. The aorta and carotids on opening show numerous transverse cracks and the lumen of these vessels is of varying diameter, an irregular thinning of the walls and shallow pouchings being present. Their walls are of the stiffness of parchment paper and many cracks are unavoidably produced by handling. The pulmonary artery is stiffened to its bifurcation. Sections of the vessels show a fatty degeneration of the smooth muscle of the media with crowding together of the elastic fibers into a compact layer. No calcification could be demonstrated.

*Rabbit 10.*—The aorta is irregularly dilated especially in the upper thoracic portion. To the touch it is stiffened throughout. The carotids are stiffened and of irregular diameter to the base of the skull. The subclavians, beginning of the large abdominal vessels, the iliacs, and about an inch of the proximal portion of the femorals are palpably stiffened. The aorta, on opening, has a scale-like appearance and shows shallow dilatations, and the abdominal aorta, carotids, subclavians, and iliacs show numerous transverse cracks. Sections of the thoracic aorta, abdominal aorta, and carotids show marked fatty degeneration of the smooth muscle of the media with crowding together of the elastic fibers into a compact layer. No calcification is present. There is considerable hemorrhage in the adventitia of the aorta.

*Rabbit 11.*—The thoracic portion of the aorta is slightly and irregularly dilated. The intima is of a dull gray color with fine closely set longitudinal striæ. No lesions are apparent in the abdominal aorta or any of the other large vessels. Sections of the arch show necrosis of the muscle in the media without calcification. There is hemorrhage into the adventitia.

*Rabbit 14.*—The arch of the aorta shows a moderate irregular dilatation. The intima of the thoracic portion is whiter than normally and shows a definite irregular thickening with longitudinal white striæ. The wall of the abdominal portion is stiff, and irregular areas of thinning are present. The carotids to the base of the skull, subclavians, iliacs, and the proximal inch of the brachials and femorals are slightly but definitely stiffened, of varying diameter, and on opening show numerous transverse cracks through the intima. Sections of the arch show an irregular marked cellular thickening of the intima without definite lesions in the media (Fig. 3). Sections of the abdominal aorta show a marked necrosis of the muscle of the media with crowding together of the elastic fibers, irregular thinning of the wall, and round cell infiltration in the adventitia. Sections of the carotid (Fig. 10) show necrosis of muscle, crowding of elastic fibers into a compact layer, and irregular thinning of the wall with small aneurysmal dilatations. No calcification could be demonstrated.



*Rabbit 17.*—An extreme diffuse lesion of the entire aorta from the aortic valves to the iliacs is present. The vessel is irregularly dilated, stiff, and definitely calcified throughout. A similar lesion involves the innominate, carotids, subclavians, large abdominal vessels, iliacs, and the brachials and femorals for a considerable distance. On palpation these vessels crackle under the finger like thin parchment paper and cracks are easily produced by bending. The pulmonary artery shows no gross lesion. Sections of the thoracic and abdominal aorta show a marked diffuse fatty degeneration and calcification of the media involving usually the inner third or half. The intima, and in places a thin layer of media overlying the zone of calcification, is necrotic and without calcium (Fig. 5). There is some hemorrhage into the adventitia. Sections of the carotids, iliacs, femorals (Fig. 11), etc., also show extensive necrosis and calcification of the media, generally diffusely involving the inner third, but in places extending nearly to the adventitia. The renal artery is similarly calcified to the kidneys. Small plates of calcification in the inner media of a large branch of the renal artery in the pelvis of the kidney are shown in Fig. 13.

*Rabbit 27.*—The lesions are of the same character, distribution, and severity as in No. 17, except that in this rabbit two small calcified plates, each about 3 mm. in diameter, are present in the pulmonary artery near the base. Sections of the arch (Fig. 4) and thoracic aorta show everywhere a wide zone of necrosis and marked calcification in the media. This calcified layer is of fairly uniform width in the arch but runs a wavy course, varying considerably in its distance below the intima. The elastic fibers themselves appear to be calcified. The intima and portion of the media overlying show considerable necrosis. In the abdominal aorta this overlying tissue, as well as the calcified material itself, in places, has sloughed off leaving deep ulcers (Figs. 7 and 8) which sometimes extend nearly to the adventitia, the latter showing hemorrhage and round cell infiltration. Small aneurysmal dilatations are numerous (Fig. 8). The carotids (Fig. 12) and iliacs also show extensive necrosis and calcification of the media.

*Rabbit 30.*—(Figs. 1 and 2.) The lesions are practically the same as in Nos. 17 and 27, but calcification of the iliacs is not so pronounced and is less extensive, and calcification of the brachials and femorals is not evident in the gross. The pulmonary artery shows a distinct fusiform dilatation (Fig. 1), the wall of which contains calcareous plates. The carotids are markedly stiffened to the base of the skull but no lesions of the vessels at the base of the brain, or in its substance, were evident in the gross or microscopically. Sections of the thoracic and abdominal aorta show similar widespread necrosis and calcification of the media with extensive and deep ulcers (Figs. 6 and 9), and hemorrhage and round cell infiltration in the adventitia. Sections of the smaller arteries also show extensive and practically uniform calcification of the media.

*Rabbit 31.*—The aorta on external inspection appears normal. The intima is whiter than normally and shows a slight but definite irregularly distributed thickening, more pronounced in the thoracic portion. The lower abdominal aorta for about 2 cm. above the iliacs is definitely stiffened and shows transverse cracks.

The pulmonary artery and branches of the aorta appear normal. Sections of the thoracic aorta show an irregular cellular thickening of the intima with no definite lesions in the media. Sections of the lower abdominal aorta show fatty degeneration of the muscular and elastic tissue of the media.

#### DISCUSSION.

The question at once arises whether the striking vascular lesion found in these rabbits is due to the treatment or preexisted as a spontaneous arteriosclerosis. That the latter occurs there can be no doubt, but in the author's experience it has been rare, only one case having been observed in a large number of rabbits whose vessels were carefully examined, and in this animal the lesion consisted of a single 2 mm. plaque of intimal thickening in the arch. Miles (18), however, reports the occurrence of spontaneous arteriosclerosis in seventeen out of forty-nine rabbits. In all these, however, the lesions were small and usually situated at the point of exit of a large vessel from the aorta. Whatever may be true as to the incidence of arteriosclerosis in rabbits no case has been reported, in so far as the author can discover, in which the lesions at all approximate in severity or extent those here reported. Even the vascular degeneration produced in rabbits by the administration of adrenalin chloride, first described by Josué (17), and since confirmed by others, but believed by some to be an independent and preexisting lesion, involves only the aorta and pulmonary artery in the experience of the writer, and it is believed that this accords with the observations of others; and even in the aorta the lesion is less severe and diffuse than that observed in these animals. The vascular lesion in these rabbits is so pronounced, in the peripheral vessels affected as well as in the aorta, that it is quite evident, after exposure of the vessel, on external inspection and even more so on palpation.

Several inconsistencies in the results obtained are, however, at once apparent on examination of the tables, as the presence of a pronounced arteriosclerosis in No. 14 and its entire absence in No. 13; also in Nos. 9 and 10. In order to produce the lesion it seems to be necessary to have a large amount of unbound toxin circulating in the blood and to produce this large doses must be injected before immunity is established. That a vascular lesion might not ultimately

result from frequently repeated small doses is not proven by these experiments, but as far as they go they tend to show that this is the case. The lack of results reported by Frothingham (11) on two rabbits as previously quoted, also gives strength to this view. The impression has also been gained that it is easier to produce the lesion in large rabbits. The differences in results are not, however, entirely explainable on the basis of dose, time, or weight of the animal, nor do individual differences in susceptibility to the toxin seem an adequate explanation.

Briefly summarized, the lesions observed consist of a fatty degeneration and necrosis of the smooth muscle of the media with subsequent crowding together of the elastic fibers into a relatively compact layer. There is also a degeneration of the elastic tissue in the media and the degenerated tissue becomes extensively calcified. In severe cases there is also a necrosis of the overlying intima and deep ulcers are formed. In certain rabbits there is a definite cellular hyperplasia of the intima which appears to be independent of medial degeneration. This will be discussed later. In none of the animals in which extensive necrosis and calcification were observed was there any inflammatory reaction in the media or hyperplasia of the overlying intima. In these cases, however, the overlying tissue was largely necrotic and sufficient time had perhaps not elapsed for reparative or compensatory processes to take place. It is quite possible that regeneration and compensatory intimal hyperplasia might ultimately occur over these medial lesions if the animals could be kept alive. The cracks mentioned as occurring both in the aorta and peripheral vessels are to a considerable extent unavoidably produced post mortem by handling the vessels. That some of these fractures, however, which extend deep into the media, existed during life is proved by the fact that in several cases small dissecting aneurysms were found in the aorta which had manifestly been produced by the entrance of blood through the rupture in the intima and inner media into the necrotic zone in the deeper media. In one case a definitely antemortem clot had formed in one of the cracks and protruded as a small thrombus into the lumen of the vessel.

It is to be noted that four of the fifteen rabbits which received diphtheria toxin alone (Table I) showed gross vascular lesions as

well as four of the seventeen rabbits which received toxin and pituitrin (Table II). It will also be noted that no calcification was demonstrated in any of the animals receiving toxin alone, while three of the animals receiving pituitrin with the toxin showed widely distributed and marked calcification. Between the two series, however, there was little difference in the gross characteristics or distribution of the lesions. The vessels in Nos. 6, 10, and 14 were almost as stiff and brittle as those in Nos. 17, 27, and 30, yet in the first three no calcification could be demonstrated either with hematoxylin or the von Kossa stain, while in the last three calcification was marked and widely distributed. Definitely palpable stiffening and a brittleness of such a degree that the vessel cannot be opened without producing many cracks extending through the intima and deep into the media are present before calcification takes place, due apparently to a degeneration of the elastic tissue which occurs in these severe cases along with the muscular degeneration. The severity of the lesion in these rabbits is shown in Fig. 10. Why calcification should occur in the fatty degenerated medial tissue of the rabbits which have received pituitrin, and not in the rabbits which have not, is difficult to explain. The two series differ only in the presence or absence of calcification, and it is felt that no emphasis should be laid on the importance of pituitrin in the production of this change. Possibly in a larger series this difference would not be so apparent. In fact, as already noted, Klotz (1) has obtained small plaques of medial calcification, limited, however, to the first part of the aorta, with diphtheria toxin alone. No case with small focal lesions was found in this series, the gross lesions being either diffuse and pronounced or entirely lacking.

The resemblance of these vascular lesions to the adrenalin type of arteriosclerosis has already been mentioned, and Klotz has called attention to the similarity of the latter lesion to the Moncheberg type of arteriosclerosis occurring in the peripheral vessels of man. In this connection it would seem appropriate to call attention to the following facts: that the lesions which have been experimentally produced with staphylococcus (Saltykow (3)), typhoid, and streptococcus (Klotz (1)) have been of the proliferative type affecting primarily and mainly the intima of the aorta and that lesions of the



peripheral vessels, except for focal lesions with staphylococcus, have not resulted; that the lesions obtained with diphtheria toxin have been degenerative and medial, closely resembling the Moncheberg type, but unlike this type in man, have been confined to the beginning of the aorta (Klotz (1) ), or have involved the entire aorta as well as to a considerable extent the peripheral vessels (present experiments); and that, on the other hand, the lesions which have been mainly associated, from clinical and postmortem observation, with infectious diseases in man have been the proliferative intimal type affecting the aorta, and an acute degeneration of the smaller arterioles in some of the organs, such as the kidneys. From such results as these here reported one is tempted to argue for an infectious or toxic origin for this peripheral type of arteriosclerosis in man, characterized by medial degeneration and calcification; and, from the results reported with staphylococcus, streptococcus, and typhoid bacillus, for a similar origin for the intimal plaques of proliferation and fatty degeneration in the aorta. Moreover, the fact that up to the present the injection of different varieties of bacteria or their toxins into rabbits has resulted in different types of arteriosclerosis at once suggests a specific etiology for these types in man. One seems, however, hardly justified in so applying these results, especially to the extent of the last conclusion. The intima of the rabbit's aorta is very thin, consisting practically of a single layer of endothelial cells lying on the internal elastic lamina of the media, and the facts above recorded may possibly be largely accounted for by anatomical structure of the vessels and strength, rather than specificity of toxin. A weaker toxin might produce proliferative rather than degenerative lesions; a stronger toxin might produce degeneration of more highly differentiated cells, as smooth muscle, before endothelial cells or fibroblasts. Since the vessel walls in these rabbits are evidently affected by toxin from the lumen rather than through the vasa vasorum we obtain in these animals, with a thin aortic intima, a medial degeneration of the aorta as well as of the peripheral vessels. If these theoretical explanations are correct we might expect to obtain with a proper strength of toxin in an animal with a thick aortic intima a proliferative intimal lesion here and a degenerative medial lesion in the smaller vessels; and in the rabbit one might hope to find a smaller dose of toxin which would produce a

proliferative intimal lesion in the aorta and a medial degeneration in the smaller vessels, which are without the dense elastic laminae of the aorta and probably more permeable. In one rabbit, No. 14, which received a relatively small dose of toxin, we find this condition present—intimal hyperplasia in the aorta (Fig. 3) and medial degeneration in the smaller vessels (Fig. 10)—thus giving a certain amount of experimental evidence for the idea that the difference in the type of lesion experimentally obtained with staphylococcus, streptococcus, and typhoid bacillus on the one hand, and diphtheria toxin on the other, are not due to specific action on a certain type of cell but to strength of toxin and anatomic structure.

Of possible interest in connection with these experiments is the work done by various authors on the effect of diphtheria toxin on adrenal function. Langlois (19), Luksch (20, 21), and Hannes (22) claimed that diphtheria toxin decreased adrenalin secretion, and Ehrmann (23) claimed that it increased it. Bonnamour and Thévenot (24) injected rabbits subcutaneously with diphtheria toxin and at the same time intravenously with adrenalin. They believe that the aortic degeneration resulting from the combined action of diphtheria toxin and adrenalin is more severe than from adrenalin alone, and that therefore the adrenalin secretion is increased. They injected no animals with diphtheria toxin alone. Tscheboksaroff (25) believes that the previous disagreement is explained by his results on dogs. He found that for 10 to 15 hours after the injection of diphtheria toxin the adrenalin secretion was increased, that in a second stage occurring 24 to 27 hours after injection the adrenalin secretion was normal, and in a third stage, 48 to 96 hours after injection, adrenalin disappeared from the blood. Abramow (26) from work on rabbits, guinea pigs, and immunized horses, concluded that under the influence of large doses of diphtheria toxin the adrenalin secretion ceased, with minimum lethal doses it decreased, and with sublethal doses and in immunization it increased. Some of these results, as well as the morphological resemblance between this and the adrenalin type of arteriosclerosis, suggest the possibility of a relationship between the two. The fact that the adrenals of these rabbits are enlarged seems no evidence of an increase in secretory function. The adrenals are also enlarged in rabbits injected with other bacteria, as in a series injected over long periods with colon bacilli by the author (27), in none of which were any lesions of the large vessels present. The enlargement of the adrenals is probably due to the storage in the cortex of fat, largely anisotropic, from the fatty degeneration produced by the toxin. If there is an increase in secretory function it is independent of this enlargement. It seems probable that the vascular lesion reported is due to the direct action of the diphtheria toxin on the vessel walls rather than through the medium of adrenalin. The latter possibility cannot, however, be absolutely dismissed.



It is not the purpose of this paper to recount the acute changes produced in various organs by diphtheria toxin, which have been previously carefully studied and described by others. It is desired, however, to mention briefly the changes in certain organs. The spleens of the arteriosclerotic rabbits which withstood the treatment longest are in marked contrast, as to size, with those of the rabbits which lived only a few days and particularly with those of rabbits, previously reported (27), which have been injected with colon bacilli. In older arteriosclerotic rabbits the organ is shrunken, being one-third to one-half the normal size; microscopically the Malpighian bodies are atrophied and there is a diffuse increase of connective tissue throughout the reticulum of the pulp. In the rabbits injected with colon bacilli the spleen is much enlarged; microscopically there is atrophy of the Malpighian bodies with a marked deposition of amyloid. In the rabbits injected with diphtheria toxin the arterioles in the spleen show no marked lesions, but that their endothelium is affected is evidenced by the fact that the lumen sometimes contains large numbers of flat, desquamated, endothelial cells.

Babes (12), experimenting on rabbits with diphtheria toxin, describes swelling, proliferation, and desquamation of the epithelium of the kidney and also of the endothelial cells of the blood vessels with the formation of hyaline masses in their lumina. Welch and Flexner (13), working on guinea pigs, rabbits, and kittens with diphtheria toxin, describe hyaline alteration of the glomerular capillaries and smaller arteries, especially in kittens. The hyaline completely filled the lumen of some capillaries. There was also fragmentation of nuclei in the glomeruli and tubular epithelium on a small scale. Frothingham (11) has described necroses of the walls of the larger vessels of the kidneys in rabbits, with fibrin deposits in the lumen and hemorrhage into the surrounding tissue. The glomeruli in cases showed partial necrosis of the capillaries of the tufts with fibrin formation in them.

The results in these rabbits confirm in the main the previous observations. In the gross the kidneys were of normal size or somewhat enlarged, they were frequently deep red, but sometimes pale and opaque. The minute dark red spots, characteristic of glomerular hemorrhages, were frequent. In a few of the more typical cases these glomerular hemorrhages, and also small opaque white spots were thickly scattered over the surface and on section were seen in the cortex. No differences were noted, either in the gross or micro-

scopically, between the kidneys of the rabbits which received toxin only and those of the rabbits which received pituitrin with the toxin unless it was that the hemorrhages were somewhat more numerous in the gross in the latter series. Even in this regard, however, the difference was not striking. Microscopically both vascular and glomerular lesions were present in nearly all the rabbits, as shown in Tables I and II, and in most cases were frequent and well marked. The vascular lesions consisted of swelling and desquamation of the endothelial cells of the arterioles and small veins with numerous lateral thromboses in these vessels. The glomeruli show necroses of the capillaries with hemorrhages into the tufts and capsular spaces and the formation of fibrinous and hyaline masses (Fig. 14). Casts and red blood corpuscles are numerous in the tubules. There is considerable pyknosis and fragmentation of the nuclei, and in some cases a swelling and apparently early cellular proliferation in the tuft. Some of the glomeruli show rather marked collections of polymorphonuclear leukocytes in and about the lesions in the tufts (Fig. 15). It seems appropriate to call particular attention to the occurrence of these focal glomerular lesions with cellular infiltration in the kidney, produced by a soluble toxin circulating in the blood, as did Welch and Flexner (14) in the case of the focal lesions in the liver. A few of the older cases show an early diffuse interstitial proliferation of connective tissue, apparently quite different from the focal scars occurring as a spontaneous lesion in rabbits. The Sudan stain shows that fatty degeneration was always present, but varying much in degree and extent. In the less severe cases the convoluted tubules only are affected; in the more severe cases the tubular epithelium is affected throughout. Moderate fatty degeneration in the tufts is frequent.

Though these lesions do not differ essentially from those previously reported, it would seem that the similarity of the nephritis to acute glomerulonephritis in man has not been sufficiently emphasized. Ophüls (28) has called attention to the importance of vascular changes in glomerulonephritis in man, and, in these rabbits, the primary lesion and essential factor in the production of the glomerular change appear to be a similar vascular damage and the result is a nephritis closely resembling the acute and subacute glomerular types in man.

It is unfortunate that it has thus far been impossible to keep a rabbit alive for more than 28 days after the injection of the large dose of toxin which seems essential for the production of the vascular lesions here reported, since a study of the subsequent morphological changes both in the vessels and in the kidneys, as well as of the functional changes resulting, would be of much interest.

#### CONCLUSIONS.

There may be produced in rabbits by the intravenous injection of large doses of diphtheria toxin a vascular degeneration involving the entire aorta, the carotids to the base of the skull, the subclavians, and iliacs, and, for a varying distance distally, the brachials, femorals, and large abdominal vessels. The first part of the pulmonary artery is sometimes affected. The lesion is practically diffuse throughout the aorta and vessels mentioned, consisting of a fatty degeneration and necrosis of the smooth muscle in a wide zone of the media and a crowding together of the elastic fibers in the region affected, resulting in an irregular thinning of the vessel walls and many small aneurysmal pouchings. In rabbits which received pituitrin with the diphtheria toxin extensive calcification occurred throughout this degenerated zone, both in the aorta and other large vessels. It is believed, however, that the pituitrin is not essential to the calcification and that if it is of any importance it is because an extreme fatty degeneration is produced more quickly in the media of the vessels when it is administered simultaneously with the toxin.

Diphtheria toxin, given in large doses intravenously, produces in the kidneys of the rabbit a pronounced vascular and parenchymatous degeneration. The former consists of a swelling and desquamation of the endothelial cells of the arterioles and small veins with the formation of fibrinous thrombi, a necrosis and thrombosis of the capillaries of the tufts with hemorrhage and the formation of fibrinous and hyaline masses, and in some of the affected glomeruli considerable collections of polymorphonuclear leukocytes.

In conclusion I wish to acknowledge my indebtedness to Dr. Ophüls for his interest and advice throughout the experiments, and particularly for his examination of the kidneys.

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## EXPLANATION OF PLATES.

## PLATE 14.

FIG. 1. Rabbit 30. The aorta and its branches. All the vessels shown were diffusely calcified with the exception of the abdominal branches of the aorta and the iliacs. In these calcification was only slightly apparent in the gross. The irregular dilatation of the upper portion of the aorta is shown ending sharply at about the level of the right renal artery. A shallow but well marked dilatation is seen on the convex surface of the descending portion of the arch just below the left subclavian artery; also a fusiform dilatation of the pulmonary artery lying just above the left auricle. The irregular thinning of the walls is apparent, particularly in the abdominal aorta and the carotids. The transverse cracks are apparent even in this external view, seen best in the abdominal aorta and right carotid.

FIG. 2. Rabbit 30. The same as Fig. 1, but with some of the vessels opened. Note the irregular dilatation of the aorta ending sharply at about the level of the right renal artery; also the circumscribed dilatation of the aorta just below the left subclavian, a similar dilatation on the posterior wall of the proximal portion of the right carotid, and the fusiform dilatation of the pulmonary artery (not opened) lying just above the left auricle. The stiffness of the vessels is evidenced by the crumpled appearance, best seen throughout the thoracic aorta, and their brittleness by the numerous transverse cracks, best shown in the photograph throughout the aorta, but also present in the other vessels.

## PLATE 15.

FIG. 3. Rabbit 14. Arch of the aorta. A marked cellular thickening of the intima is seen, without apparent lesions in the media. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

FIG. 4. Rabbit 27. Arch of the aorta. A wavy zone of marked calcification in the media is present, of fairly uniform width but varying in depth below the intima. There is considerable necrosis with lack of nuclear staining in the overlying intima and media. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

FIG. 5. Rabbit 17. Thoracic aorta. There is a zone of marked calcification in the upper media with necrosis of the intima and overlying media. On the left, calcified spicules have broken through and project into the lumen. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

## PLATE 16.

FIG. 6. Rabbit 30. Abdominal aorta. There is extensive calcification in the media, complete sloughing of the intima with ulcerations extending deeply into the calcified areas of the media, and a cellular thickening with round cell infiltration in the adventitia. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

FIG. 7. Rabbit 27. Abdominal aorta. The wall of the vessel is largely necrotic and extensively calcified, with the exception of a narrow zone next to the adventitia. In the center of the photograph the media is of about normal thickness, the intima has sloughed, and two zones of calcification are seen, one on the surface and one in the deep media, the intervening medial tissue being necrotic with little nuclear staining. At either side are deep ulcers extending almost through the media. There is hemorrhage in the adventitia. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

FIG. 8. Rabbit 27. Abdominal aorta. A shallow aneurysmal dilatation is present. Otherwise the condition is similar to that in Fig. 7. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

## PLATE 17.

FIG. 9. Rabbit 30. Abdominal aorta. Extensive necrosis and calcification of the media with broad shallow ulcers. Note the marked round cell infiltration in the adventitia. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

FIG. 10. Rabbit 14. Cross-section of the carotid artery. Necrosis of the muscle is seen throughout a narrow zone of fairly uniform width in the upper media with crowding together of the elastic fibers into a compact layer. There is an irregular thinning of the wall with the formation of shallow pouchings.



There is no calcification. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

FIG. 11. Rabbit 17. Cross-section of the femoral artery. There is a zone of calcification in the upper media and at the right a second area of calcification and softening in the deeper media. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

## PLATE 18.

FIG. 12. Rabbit 27. Cross-section of the carotid artery showing calcified plates in the upper media. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

FIG. 13. Rabbit 17. Cross-section of a branch of the renal artery in the pelvis of the kidney, showing small calcified areas in the upper media. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

## PLATE 19.

FIG. 14. Rabbit 10. Kidney. Lesions are present in all the glomeruli, consisting of partial necrosis of the tufts with the formation of fibrinous and hyaline masses. Many of the tubules contain casts. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{3}{8}$ , oc. 1.

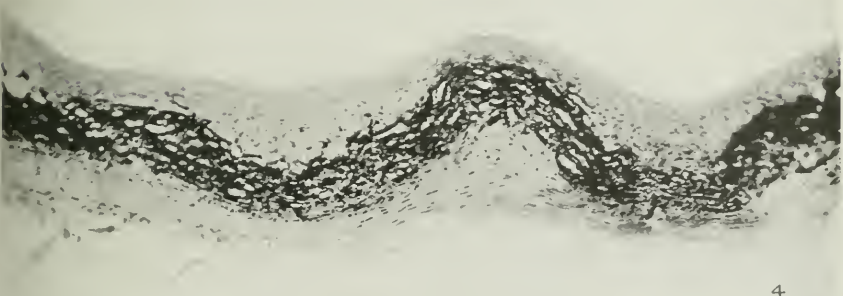
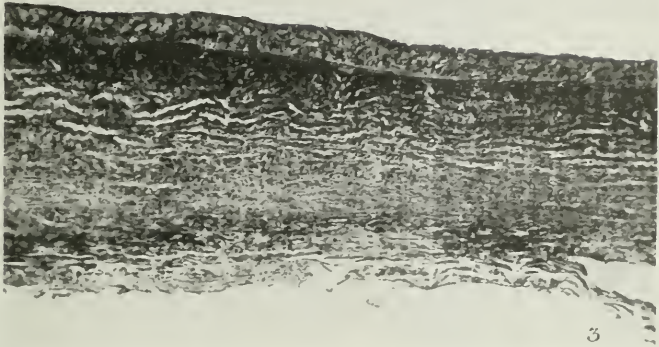
FIG. 15. Rabbit 27. High power of a typical kidney glomerulus, showing degeneration and necrosis of the tuft with hemorrhage and the formation of hyaline and fibrinous masses and a rich infiltration with polymorphonuclear leukocytes. Microphotograph. Hematoxylin and Van Gieson's stain. Bausch and Lomb obj.  $\frac{1}{8}$ , oc. 1.





(Bailey: Arteriosclerosis and Glomerulonephritis.)

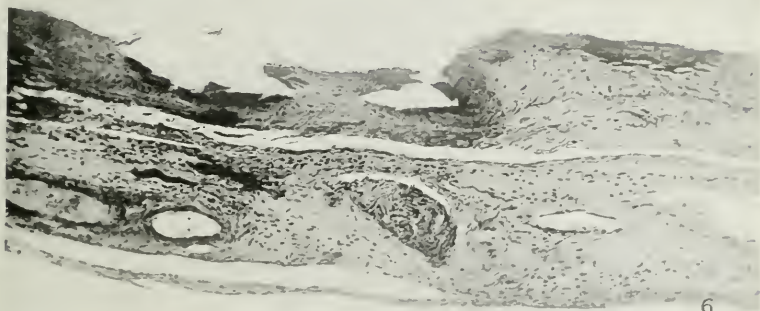




(Bailey: Arteriosclerosis and Glomerulonephritis)







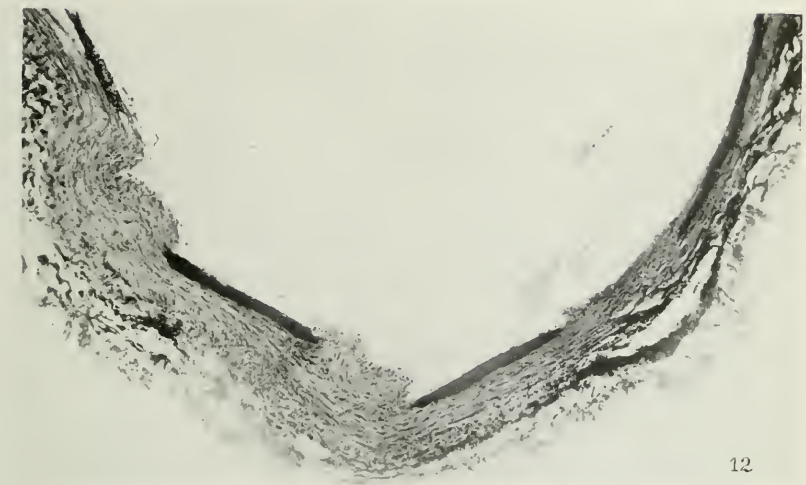
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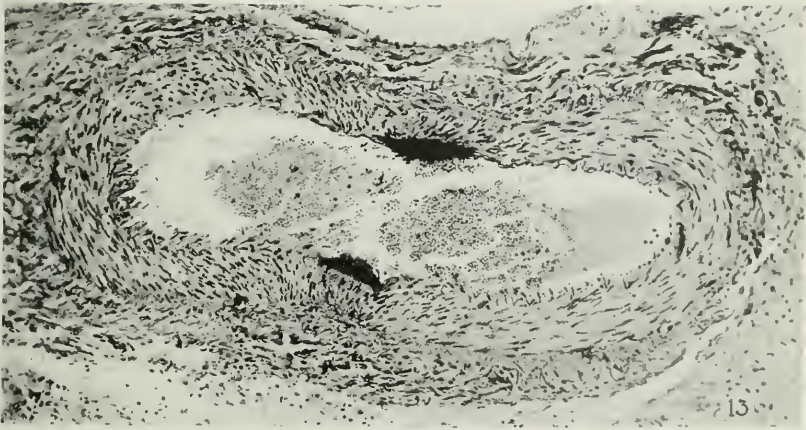


(Bailey: Arteriosclerosis and Glomerulonephritis )





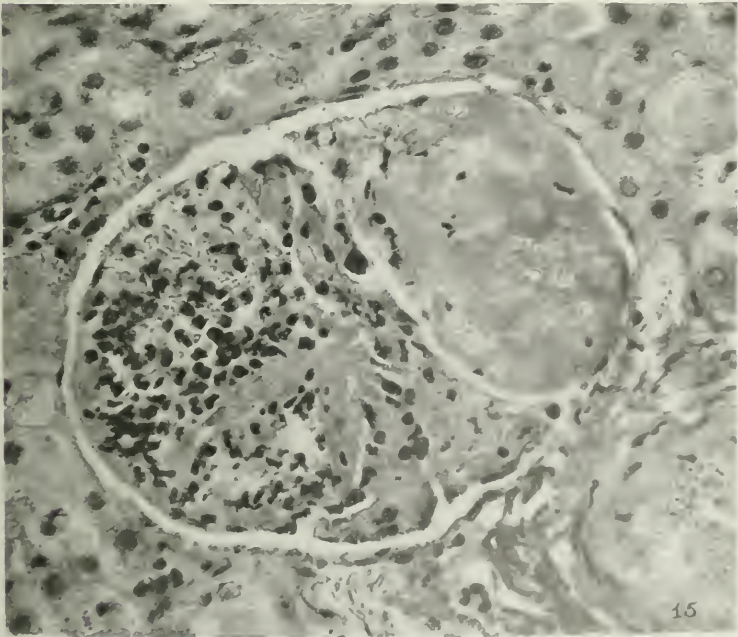
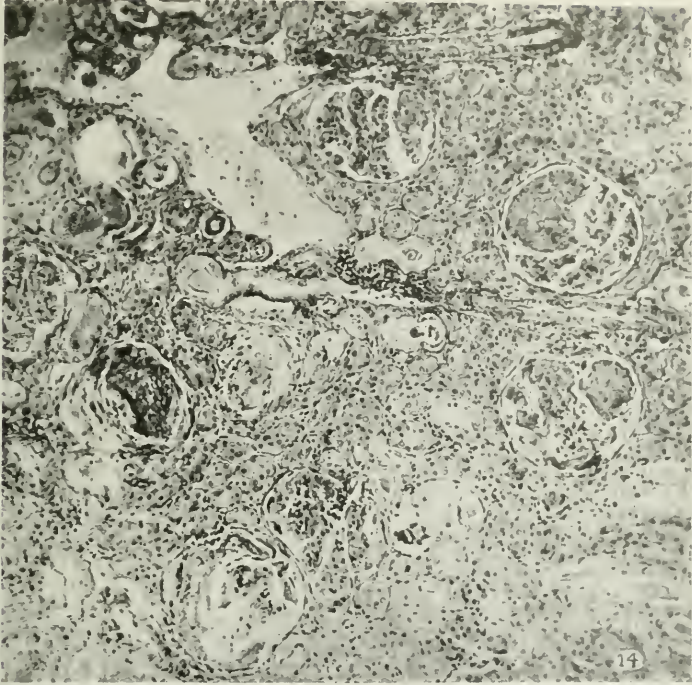
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13







(Bailey: Arteriosclerosis and Glomerulonephritis.)



# EXTIRPATION OF THE THYMUS IN THE GUINEA PIG.

By EDWARDS A. PARK, M.D.

(From the Department of Pediatrics of the Johns Hopkins University, Baltimore.)

PLATES 20 AND 21.

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## HISTORICAL.

In 1845 Restelli attempted to determine the function of the thymus by extirpating the gland from dogs, sheep, and calves. Since then more than twenty-five investigators have performed many series of extirpation experiments and extended their operations to more than fourteen species of animals, without obtaining results of sufficient agreement to bring the question of the effects produced by thymus extirpation to a final settlement. Some have found that removal of the thymus is without effect (Fischl, Pappenheimer, Nordmann, and others), and inferred that the thymus has either no function in postfetal life (Fischl) or none that can be demonstrated. Others have seen changes of a transitory character develop, and consequently have thought that the thymus exercises a transitory function corresponding to its own life history. Most prominent among these is Basch, whose experiments on dogs have been the models for subsequent investigators. Still others (Abelous and Billard, Matti, and Klose and Vogt) have determined that removal of the thymus is followed by symptoms and pathological changes of a profound character, culminating in death; and they have drawn the conclusion that the thymus is essential to life and assign to it an importance comparable to that of the adrenal, parathyroids, and pancreas.

The animals that have been used for extirpation experiments are the dog, rabbit, rat, guinea pig, frog (also tadpole), cat, fowl, pigeon, goat, sheep, calf, pig, and monkey; but some species are better adapted for thymus extirpation experiments than others. Basch is the authority for the frequently quoted statement that herbivorous animals are poor-subjects, because very early in these animals bone has a high calcium content, which acts in a protective manner against the influences that result from the cessation of thymus function. It is well known, however, that rickets occurs spontaneously in herbivorous animals (Jost and Koch), and numerous investigators have reported changes in the skeleton of the rabbit following thymectomy, and one (Klose) has obtained rachitic-like changes in goats. But no one has succeeded in producing alterations in the skeleton of the guinea pig by thymus extirpation—unless the slight retardation in growth, noted by Soli,

is to be regarded in that light. If Basch's observation is true, that deprivation of the thymus is less apt to be followed by bone changes in herbivora than in omnivora or carnivora, another explanation is that extirpation of the thymus in the former is almost invariably incomplete.

In order to make clear the anatomical relations of the thymus in the guinea pig which distinguish it from all other mammals and make complete removal impossible, it is first necessary briefly to trace the development of the thymus not only in the guinea pig but in other mammals.

*Development of the Thymus in the Higher Mammals, with Especial Reference to Its Development in the Guinea Pig.*

The higher mammals may be divided into three groups, according to the situation of the thymus (Hammar). In the first group the thymus is chiefly or entirely in the thorax; in the second, in both thorax and neck; and in the third, in the neck alone.

The thymus of most of the higher vertebrates is derived from the third pharyngeal pouch alone or from the third and fourth pouches, and is therefore purely entodermal in origin. The thymus of the swine (Zotterman, Badertscher) and of the mole (Schaffer and Rabl), on the other hand, takes origin not only from the third pharyngeal pouch but also from the ductus precervicalis medialis, a derivative of the ectoderm, and the thymus of the guinea pig also has a twofold origin from ectoderm and entoderm, as will be pointed out later.

The mammalian thymus appears very early as a cylindrical outgrowth in a ventral and mesial direction from the ventral diverticulum of the third pharyngeal pouch. Simultaneously with the appearance of this thymus anlage, called thymus III, there occurs a proliferation of the epithelium of the dorsal diverticulum and adjacent parts of the pouch which soon undergoes histological differentiation into the anlage of the larger of the two parathyroids, parathyroid III.

While thymus III and parathyroid III are forming from the ventral and dorsal diverticula of the pouch, the mesial portion, roughly that part of it between these structures and the pharynx, called the ductus pharyngobranchialis, narrows, atrophies, and disappears



altogether, so that thymus III and parathyroid III are freed from their connection with the pharynx, becoming independent structures. The thymus anlage elongates and is now termed the thymus cord.

In the species constituting the first group, of which man and the dog are representative, the caudal end of the thymus cord thickens and the cranial end becomes thinner. The entire cord then begins a migration in a caudal direction, its descent being associated with that of the great vessels and heart, and since the thick caudal portion moves at a more rapid rate than the thin cranial portion, the latter becomes drawn out into a thin strand. The thick caudal portion, continuing its migration, enters the thorax and, joining its fellow of the opposite side, forms the thoracic portion of the thymus. The thin strand representing the cranial portion of the cord atrophies and entirely disappears, except the lowermost part, which persists as a continuation of the thoracic portion into the base of the neck, forming the cervical process, or cornu of the completely developed gland. Parathyroid III, which remains connected with the upper end of the thymus cord, is carried caudally with the latter, but halts near the lower end of the thyroid anlage, assuming its permanent position near the lower pole of the thyroid.

In the second group of animals, which have both neck and breast thymus (the hoofed animals are examples), the caudal portion of the thymus cord enlarges and enters the thorax to form the thoracic thymus, exactly as has been described for the first group. But the remainder of the cord does not atrophy and disappear; indeed the cranial end of it hypertrophies, forming an enlargement, known as the head of the thymus, and in certain members of the group, at least (ox, horse, goat, and swine) moves in a cranial direction with the growth of the neck or, according to some embryologists, is caught in the loop of the hypoglossal nerve and drawn towards the cranium to a position opposite the bifurcation of the great vessels, well above its point of origin. Shortly before birth, or during the first year, the stem of the thymus, connecting cervical and thoracic portions, disappears, so that the two parts of the gland become separate.

In the guinea pig,<sup>1</sup> which forms the third group, the thymus anlage

<sup>1</sup> The development of the thymus in the guinea pig has been investigated by Maximow and Ruben. The account of its development is based on the work of Ruben.

develops from the whole of the third pharyngeal pouch with the exception of those parts of it which form parathyroid III and the neck. When set free together with parathyroid III from its connection with the pharynx through atrophy of the neck of the pouch, it does not elongate as in those animals destined to have a thoracic thymus, but assuming a rounded or oval form remains essentially in its original position. According to Ruben, the loop of the hypoglossal nerve cuts in between parathyroid III and the thymus mass, separating the former from the latter without carrying the latter upwards. A portion of the ectodermal precervical vesicle early becomes incorporated.

In certain species of animals a thymus anlage, first described by Groschuff, develops also at the fourth pharyngeal pouch together with the parathyroid of that pouch, and is known from its origin as thymus IV, or thymus metamere IV. When the neck of the fourth pharyngeal pouch disappears, thymus IV and parathyroid IV in their turn migrate, but in a mesial direction, to the lateral portion of the thyroid anlage, where they take their permanent position together. In several species parathyroid IV regularly becomes incorporated in the substance of the thyroid, and thymus IV also, if present. Thymus IV has been found in the cat, ox, sheep, horse, goat, rat, rabbit, bat, and more rarely in man and the dog (Groschuff, Kohn). In the guinea pig, however, it does not develop, and even parathyroid IV is frequently rudimentary or present only on one side, or is even absent altogether (Ruben).

Thus the thymus in the guinea pig, unlike the thymus in other mammals, remains a purely cervical organ<sup>2</sup> (Ruben, Maximow) and does not possess the accessory lobe derived from the fourth pharyngeal pouch so frequently seen in other species. It would seem, therefore, as if the guinea pig should be especially adapted for complete extirpation of the thymus. That this is not the case, however, will be shown later.

<sup>2</sup> Though the thymus of the mole has an apparent resemblance to that of the guinea pig in that it also is limited to the neck, the actual development in the two animals is different, because a thoracic thymus actually forms in the mole and subsequently atrophies, while in the guinea pig none ever forms.



*Results of Previous Investigators Following Thymus Extirpation in the Guinea Pig.*

At least four investigators have used the guinea pig for the study of the effects of thymectomy. Basch states that he has performed thymectomy experiments on guinea pigs, but he has left no record of them so far as can be found.

Vincent, in 1903, removed the thymus from a series of guinea pigs aged from 10 days to 1 month, but was unable to discover any effect on growth or any change in the blood.

Paton and Goodall, in the following year, performed extirpation experiments on the guinea pig. They found that deprivation of the thymus was followed by a decrease in the number of lymphocytes for about 2 months without coincident alteration in the other elements of the blood, but they observed no disturbance in growth and no variation of any kind from the normal.

In 1905, Paton published a new set of thymectomy experiments on guinea pigs, in which special study was made of the effects produced on the genital organs. His experimental material comprised twenty-four guinea pigs, 1 day to 1½ months old at the time thymectomy was performed, and killed at varying periods after operation. The control animals were selected from the same litters when possible, but when this was impossible (and he does not indicate how often this was) they were matched with the operative animals according to weight. Inasmuch as he had no record of the dates of birth of his animals he was compelled to estimate their ages from their weights. Since he found that sexual maturity in the guinea pig occurs at about the 3rd month, when the animal has reached a weight of approximately 300 gm., he assumed in his experiments that animals under 300 gm. were under 3 months of age and sexually immature, while animals over 300 gm. were over 3 months of age and sexually mature. The first group of six guinea pigs (there were six controls) weighed between 75 and 145 gm. at the time of operation and were allowed to live from 2 to 6 weeks; they were killed at such times as to make the final weight of all members of the group lie between 100 and 200 gm. The average postmortem weight of the operative animals was 165 gm., of the controls 152 gm.; of the testes and epididymes of the operative animals, 0.23 gm.; of the testes and epididymes of the controls, 0.18 gm. The second group of sixteen animals (there were fourteen controls) weighed from 84 to 280 gm. when the thymus was removed, and were killed at the end of 4 weeks—in two instances 8 weeks—when the weights of all were between 200 and 300 gm. The average final weight of the operative animals of this group slightly exceeded that of the controls, but the average weight of the testes and epididymes of the thymectomized animals was 0.88 gm., while that of the controls was but 0.60 gm. The two groups just mentioned comprised the immature material of Paton's experiments. The third group, composed of but two operative animals (there were three controls), represented his entire mature material. One of the two, weighing 250 gm. at the operation, was killed at the end of 3 weeks,

weighing 310 gm. The other, weighing 280 gm. when operated upon, was allowed to live only 1 week. The testes, with epididymes attached, of these two animals weighed almost the same (1.24 gm.) as those of the three controls (1.33 gm.). Paton was unable to show that the loss of the thymus affected in any way the time of conception of female guinea pigs.

Though it seems questionable whether Paton could draw definite conclusions from his experiments, except perhaps such general ones as that removal of the thymus in the guinea pig is compatible with life, or that it does not produce any very gross changes, he drew the specific conclusion that the thymus inhibits the growth of the testes in immature but not in mature animals; and since Calzolari, Henderson, and others had already established the fact that the thymus continues to persist uninvoluted in animals castrated before maturity, he put forward the theory that prior to the period of sexual maturity thymus and sex glands act antagonistically, each exerting an inhibitory influence on the other.

In the same year (1905) and again in 1909 Soli performed thymectomy experiments on guinea pigs. The major part of his work, however, was done on fowls, in which he discovered that extirpation of the thymus resulted in well marked retardation in growth and development of the testes. He obtained similar results in the majority of fifteen guinea pigs, although they were much less marked than in the case of the fowls. Although Soli's results in guinea pigs appear to contradict the findings of Paton, they do so only to a limited extent, for all Soli's guinea pigs with the exception of two weighed more than 300 gm. when killed (Paton). (The maximum weight for immature animals in Paton's experiments was 300 gm.). The testes of Soli's two thymectomized guinea pigs, which weighed under 300 gm., were, however, slightly lighter than those of the controls.

In 1911 Paton returned to the problem of the relationship of thymic and testicular functions. Was it not possible that each organ exercised a primary, independent, stimulating influence on growth rather than an inhibitory influence on the other, as according to the theory he had previously advanced? To put this new hypothesis to the test, he first determined in a series of four guinea pigs and controls that castration alone before sexual maturity, *i.e.*, in animals under 300 gm., did not affect the weight. He next found by combined extirpation of both thymus and testes in a series of sixteen guinea pigs under 300 gm., that retardation in the growth of the animal measured in terms of weight did occur. Since his previous experimental work had shown that loss of the thymus alone had no influence on growth, he regarded the chain of experimental evidence necessary for the new hypothesis as complete. His final conception of the relationship of the two organs to each other was, therefore, that each stimulated growth in the immature animal independently of the other and consequently that the hypertrophy of the testes, when the thymus was removed, and the continued persistence of the thymus when the testes were removed, in sexually immature animals were compensatory in nature. On performing similar experiments with female guinea pigs he was unable to produce changes corresponding to those in the male.

It should be added that Paton used controls from "the same batches" of animals, and again that he had recourse to rough estimations of the ages from the weights.

Halnan and Marshall are the most recent investigators to attack the thymus problem in the guinea pig. They found that its removal in young guinea pigs, most of which weighed about 150 gm. did not affect growth, and that castration led to an arrested atrophy and subsequent hypertrophy of the thymus, and, more important, as bearing on Paton's work, that simultaneous removal of the testes and thymus did not affect the growth of immature animals, and thymectomy was not followed by hypertrophy of the testes. Thus they entirely failed to confirm the results of Paton. Their paper concludes with a note from Yule showing by methods of statistical analysis that the differences which Paton found in the testes of thymectomized and control guinea pigs were not greater than could be explained on the basis of chance variation.

Paton and Goodall remark: "The chief obstacle to the investigation of the functions of the thymus is the difficulty of its removal on account of the situation of part of it, in most animals, in the thorax. We were fortunate enough to begin our investigations upon guinea-pigs and we found that in these animals the structure is entirely cervical." Halnan and Marshall speak of thymectomy in the guinea pig as a simple operation. Klose and Vogt, on the other hand, describe thymectomy in the guinea pig as a dangerous operation which must be done in two stages and frequently causes death from hemorrhage. The writer selected the guinea pig for his experiments.

#### EXPERIMENTAL.

The operation for the removal of the two main lobes of the thymus in the guinea pig is simple, owing to their superficial position, for they lie one on either side of the median line of the neck, just beneath the platysma and deep fascia about midway between the tip of the lower jaw and the upper end of the thorax, a little in front of, but almost opposite the angle of the jaw. A median incision about 0.5 cm. long, with its middle point a little anterior to the angle of the jaw, is carried to the deep fascia, and when the edges of the wound are pulled apart the two flat, oval-shaped, granular-looking lobes of the thymus can be plainly seen shining through, having a paler color than the pink salivary glands which lie near them. Each lobe in the well nourished new-born guinea pig weighing 100 gm. measures from 10 to 15 by 7 to 10 mm. The deep fascia is then incised and each lobe in turn lifted up and freed from the salivary glands by blunt dissection. As these latter are pushed away a sheet of transparent fascia

is exposed, connecting the thymus with the neck, and in this sheet of fascia two blood vessels, larger than the rest, can be seen, one entering the lobe a little behind the anterior pole, the other a short distance in front of the posterior pole. In the earlier experiments these vessels were carefully tied off, but in the later experiments they were burned through, together with the fascial sheet, by means of a hot platinum wire. At this point in the operation great care is necessary not to leave accessory lobes of thymus, which become visible only as the main lobes are lifted up, lying near but unattached to the main lobe or close to the salivary gland, in the fascial sheet mentioned. If the main lobe of the thymus is removed first with the expectation of excising the accessory lobes later, the latter are found to fall back into the cavity of the wound the moment tension is released, and are lost. It is necessary therefore to cut or burn through the fascial sheet as far away from the main lobe as possible in order to secure the accessory lobes with the main lobes, and often to remove also fairly large parts of the salivary glands, if the latter cannot be easily separated. Frequently as many as two accessory lobes are present on each side, not larger than pin heads.

*The Discovery, by Serial Section, of Accessory Lobes of the Thymus in Close Relation to the Parathyroids.*

The operations were conducted under ether anesthesia. Little preparation aside from shaving the operative field and cleansing with alcohol was necessary, for the animals never became infected. As soon as they had recovered from the anesthesia they appeared to be well. A number of the guinea pigs died a short time after the operation but not as the result of it, for the mortality was equally high among the controls.

The animals were kept under observation for variable lengths of time, and were then killed. At autopsy the tissues of the neck of all the thymectomized animals were taken out *en masse* to be studied in serial section for thymus rests. As the block of tissue was removed from each animal it was turned over on its under surface, and first the thyroid, then the larynx and trachea, were dissected away, the former for finer histological study than would otherwise have been possible, the latter to facilitate serial section cutting. Great care



was taken to carry away with the trachea and thyroid as little other tissue as possible.

When the serial sections from the cervical tissues of nine of the thymectomized guinea pigs were studied, it was found that thymus rests near the parathyroid were present in six of them; in the remaining three no thymus tissue could be discovered. In these three thymus-free animals, however, no parathyroid tissue could be found, and inasmuch as the only conceivable explanation for the absence of the parathyroids was that they had been lost when the thyroids and trachea were removed, the possibility became apparent that accessory lobes of thymus lying near the parathyroid might also have been there and been lost with the latter. In the cervical tissues of two of the animals which had thymus rests near the parathyroid, accessory thymus tissue at some distance from the parathyroids was also found.

Since it seemed scarcely worth while to investigate the mutilated cervical tissues of the remaining thymectomized guinea pigs in order to throw further light on the occurrence of accessory lobes of thymus tissue, the intact cervical tissues, including trachea and thyroid, of five additional guinea pigs were examined in serial section. Of these five guinea pigs, four came from the same litter, and of the four, two had been freshly thymectomized. In all five animals accessory lobes were found in proximity to the parathyroids, and in three of them additional accessory lobes not connected with the parathyroids and too far removed from the main body of the thymus to have been reached at operation.

Although the number of guinea pigs examined is too small to permit any general conclusions, it seems probable that accessory lobes of thymus occurring in relation to the parathyroids are usual in the guinea pig and that other accessory lobes too remote from the main lobes of the thymus for removal with the latter are not uncommon.

*The Relations and Characters of the Accessory Lobes of the Thymus Found in the Guinea Pig after Supposed Complete Thymectomy.*

The accessory thymus tissue which was found in relation to the parathyroids was in the form of lobes having well defined cortex and medulla, and in short a histological appearance identical with that of the main lobes. In some instances only a single lobe could be found

in relation to a parathyroid, but in other instances there appeared to be several separate lobes clustered around it; though when certain sections seemed to indicate the presence of several separate masses of thymus, the study of the other sections sometimes showed that in reality a single lobulated mass of thymus tissue existed. In some of the animals the accessory lobe of the thymus, or the largest of them if several existed, was two to three times the size of the parathyroid, but in a number of the animals the accessory thymus lobe was about the same size as the parathyroid or even smaller. In at least half the animals thymus tissue and parathyroid were fused together, or at least one passed directly into the other. In one animal the parathyroid on the right side was drawn out into a thin strand which was much longer than the thyroid and extended considerably in front of it; it terminated in a small thymus lobe, so shaped that it would have been regarded as the continuation of the parathyroid but for its different structure (Fig. 2). In sections from another animal, thymus and thyroid tissue were so intermingled at one point that there was great difficulty in distinguishing them, while at another point in the sections an island of thymus surrounded by parathyroid tissue could be seen (Figs. 3 and 4). In the animals in which thymus tissue did not actually join parathyroid, it was situated close to it, never much farther away than the thickness of the parathyroid.

Of course in the mutilated cervical tissues of the six thymectomized animals first studied, it was not possible to determine more than the size and relations of the accessory lobes with reference to the accompanying parathyroid, but it was possible to obtain additional and more precise information from the study of the intact cervical tissues of the five guinea pigs killed for that purpose. In them it was found that the accessory lobes of thymus occurred on both sides in only three animals, though they occurred on one side in all five, as already stated. It was also clear that the parathyroid which the accessory lobes accompanied was the one from the third pouch. The identity of this parathyroid could be determined (1) by its larger size, and (2) by its position with reference to the thyroid, for in all five animals it lay on the outer side of the thyroid, in three instances separated some distance from the latter. In most of the animals it was in proximity to a comparatively large blood vessel, supposed to be a thy-



roid artery. In one animal the accessory lobe lay on one side of this vessel, the parathyroid on the other. On the other hand, parathyroid IV, which was much smaller than parathyroid III, lay close to the thyroid on its inner surface in all but one instance, and in three animals was partially embedded in its substance. It was absent on one side in three animals, and so small as to be barely visible in one. Accessory lobes of thymus were not found in association with this parathyroid (Fig. 1).

In this connection it is worth noting that no constant relationship in the positions of the two parathyroids in the cranial caudal direction existed. In some of the guinea pigs parathyroid III was anterior, in others posterior to parathyroid IV. In one animal parathyroid IV was in front of parathyroid III on one side and behind it on the other. The commonest arrangement seemed to be for parathyroid III to lie near the anterior end of the thyroid and parathyroid IV near the middle or posterior part. In the mammals in which the thymus migrates caudally, it will be remembered that parathyroid III is carried caudally with the thymus so that it comes to be the more caudally situated of the two parathyroids. The absence of this relationship in the guinea pig is easily explained by the failure of the thymus to migrate in that animal.

In addition to the accessory lobes already described as occurring in intimate relationship with the parathyroids, accessory lobes were found in two of the animals at some distance from the parathyroid in the neighborhood of the main thymus lobes, but they were probably too far separated from them to have been removed at operation.

The existence of accessory lobes of thymus derived from the third pharyngeal pouch lying in close relation to the parathyroids, has never before been described in the guinea pig, as far as the writer is aware, but their occurrence in other species has been known for some time. They are usually present in the rabbit, cat, and rat (Kohn), and have been found in the dog and in man (Ammann, Kürsteiner). The study of the development of the thymus makes their presence and also their close connection with parathyroid III easy to understand, for, as already pointed out, thymus and parathyroid take origin coincidentally in the closest proximity from the third pharyngeal pouch, are freed at the same moment by the atrophy of the neck of the pouch, and, if

migration occurs, migrate with each other. To explain the presence, therefore, of an accessory lobe of thymus in close connection with the parathyroid it is necessary merely to suppose that a part of the original thymus anlage adjacent to parathyroid III adhered to the latter at the time of their separation. If the theory of Ruben is correct that in the guinea pig parathyroid III is separated from the thymus mass through the agency of the loop of the hypoglossal nerve in its passage upward, it would be necessary simply to assume that the nerve had cut off a small portion of the main mass of the thymus with the parathyroid instead of accurately dividing the one from the other.

The practical significance of the discovery of these minute accessory lobes of thymus in such intimate relation to the parathyroids is that the thymus can only rarely be completely extirpated in the guinea pig. Almost all, if not all, thymectomies in this animal have been, therefore, partial thymectomies, and the positive experimental findings of Soli and Paton must be interpreted in that light.

*The Value of Incomplete Extirpation of the Thymus as a Means of Determining Thymus Function.*

The question naturally suggests itself whether incomplete extirpations of the thymus have any value as a means for determining thymus function. Since it is well established that reduction of certain organs of internal secretion, for instance the adrenal, thyroid, and parathyroid, to minute rests causes well marked signs of insufficiency, it might reasonably be supposed that sudden reduction of the thymus to a small rest in a newly born animal would make itself known, if it plays the important part in metabolism attributed to it by Matti and Klose. And the literature contains considerable evidence that this is the case. Some of Basch's<sup>3</sup> extirpations in dogs seem to have been incomplete. Fulci obtained marked rachitic changes in a rabbit by means of thymectomy, although a thymus rest almost as large as the normal gland was found at autopsy. Flesch discovered that incompletely thymectomized rats died in a manner indistinguishable from the completely thymectomized animals, with but a single excep-

<sup>3</sup> See Basch, K., *Wien. klin. Woch.*, 1903, xvi, 893.

tion. Though Klose and Vogt criticize the validity of a large part of the negative experiments of others by assuming that the thymectomies were incomplete, they accept the experimental work of those who have obtained results similar to their own, passing over the fact that the larger part of these thymectomies would be shown to be incomplete if subjected to the analysis which they applied to the other experiments. But recently Klose has reported rachitic changes in swine and goats induced by thymectomy, although complete thymectomy is impossible in both species, as he admits. To the group of investigators who have obtained positive results by means of partial thymectomy may now be added the names of Paton and Soli.

Doubtless many of the extirpations of the thymus which have been regarded as complete and reported as such in the literature in reality were partial extirpations, for it is impossible to be certain at the time of operation that a branching, friable organ like the thymus of the larger animals that have been used for extirpation experiments has been completely dissected out of the thorax and neck, especially considering the possibility of the accessory lobes. Likewise the failure to discover thymus rests at autopsy by inspection of the neck and thorax or microscopical section of bits of thymus tissue falls far short of proof that no thymus rests were actually there. The painstaking researches of Grosser and Betke have shown that in children accessory thymus lobes occur in the neck with a frequency never before suspected. If the thoracic and cervical tissues of larger animals that have been thymectomized, such as the dog and rabbit, could be studied in serial section, as was done by Pappenheimer in the rat, and by the writer in the guinea pig, it might be shown that complete thymectomy was rarely accomplished. Klose's statement that the most minute thymus rest left at operation regenerates until it reaches a size approximating that of the intact gland is not true. The literature seems to furnish, therefore, numerous instances of the effectiveness of partial thymectomy, and particularly, when the positive results of the partial thymectomies in guinea pigs of Paton and Soli are considered, justifies the publication of the following partial thymectomy experiments. But the actual problem is not whether partial thymectomy can produce results identical with complete thymectomy, but whether thymectomy, partial or complete, actually causes the changes which have been ascribed to it.

*Results of Thymus Extirpation.*

Fifty-five guinea pigs were thymectomized in the manner described, on the 1st, 2nd, 3rd, or 4th days of life, and in all but two instances were controlled from the same litter and sex. But since the mortality among such young animals was high, from trampling, inanition, and other causes, a large number of the experiments were interrupted at the outset. If the control died at the beginning of an experiment a guinea pig differing only a day or two in age was supplied in its place from another litter and the experiment continued. Later two epidemics of dysentery were encountered, which caused a number of fatalities and still further reduced the material, leaving eighteen thymectomized and sixteen control guinea pigs, which form the basis of this report. Twelve of the eighteen thymectomized animals had their original controls, and form, therefore, a complete series for the study of the effects of thymectomy from all standpoints. The remaining six thymectomized and four control animals lost their mates too late in the course of the experiments for anything to be gained by pairing them with each other or with fresh animals from other litters. Accordingly the six operative animals, with the four normal animals, are grouped separately as imperfect experiments which have value for the histological study of the organs but not for the study of the effects of thymectomy on growth or development.

All the animals were confined, in pairs, in wire cages about  $1\frac{1}{2}$  feet long by 1 foot broad in a well lighted room, and were fed a diet of greens and oats each once daily. They were inspected frequently and systematically examined at the weighing times, which appear on the weight charts, for the various changes which have been reported in animals deprived of the thymus. One operative animal and two controls died spontaneously at the conclusion of the experiment, but the others were killed with ether. The bodies of all were autopsied at once, the stomach and intestines with their contents removed and weighed separately in order to determine the actual body weights of the animals (minus the weight of the food), and the weights of the different organs were taken. The tissues were preserved in formalin and the sections stained with hematoxylin and eosin. The growth of the thymectomized animals was normal.



The weight curves of the twelve operative animals and their controls were compared in pairs, but without demonstrating any preponderant variation at any stage of development, although there was considerable variation in the weight of the pairs of animals considered separately. In some unpublished experiments by Auchincloss and the writer guinea pigs were obtained prematurely by Cæsarean sections and thymectomized at once without causing any alteration in growth or development. The series of weights in two of these experiments are appended to the table of weights of the animals of the writer's own experiments (Table I).

The strength and activity of the operative animals also appeared to be normal. As the two guinea pigs constituting each experiment were caged together, it was possible to form some idea of their relative strength by observing which drove about the other. In some instances it was the control, in others the operative animal that had the mastery.

The evidence of sexual maturity appeared to be unaffected by thymectomy. This observation was made in the experiments of Auchincloss and the writer already referred to, in which it was found that the testes of thymectomized and control males descended at about the same time and that several pairs of males and females thymectomized soon after birth, caged separately, and allowed to breed in competition with control pairs of intact males and females of the same age, produced young at about the same age.

No trophic disturbances of the hair or abnormality in the growth of the teeth were noted in the operative animals.

At autopsy the fat appeared to be equally distributed in both sets of animals, and the viscera of the thymectomized animals did not differ in their gross appearance from the viscera of the controls. The weights of the animals and of their organs are given in Table II. Although there is exhibited the greatest individual variation in the weights of the organs, there seem to be no constant differences in the two groups. The most interesting feature brought out in the table is the relatively large weight of the gastric and intestinal contents in the guinea pig. It will be noted that the excised stomach and intestines and contents of the guinea pigs—they were killed at various times of the day—weighed between 38 and 160 gm., with an average

TABLE I.  
*Weight of Thymectomized and Control Guinea Pigs.*

No. and sex.	Age at op.	Age at death.		Killed or died.	Record of weights in gm.							
		days	mos. days		1915 Mar. 2	1915 Mar. 9	1915 Mar. 23	1915 Apr. 6	1915 Apr. 27	1915 May 11	1915 May 17	
1* ♀	1	2	15	Died.	50	90	140	190	220	210	190	
1C ♀	1	2	15	"	40	80	150	250	185	190	140	
					1915	1915	1915	1915	1915	1915	1915	
2* ♀	1	2	15	Killed.	60	120	165	150	200	290	266	
2C ♀	1	2	15	Died.	40	80	150	250	185	190	140	
					1914	1914	1914	1914	1914	1914	1914	
3* ♀	4	6	20	Killed.	158	270	540	480	560	560		
3C ♀	4	6	20	"	122	200	425	300	420	500		
					1914	1914	1914	1914	1914	1914	1914	
4* ♀	3	7	25	"	121	132	320	490	540	525	580	
4C ♀	3	7	25	"	115	129	370	460	550	505	520	
					1914	1914	1914	1914	1914	1914	530	
5* ♀	3	6	7	"	120	190	265	380	290	375	385	
5C ♀	3	6	7	"	122	195	285	400	340	445	450	
					1914	1914	1914	1914	1914	1914	1914	
6 ♀	3	6	19	"	132	215	360	435	345	485	552	
6C ♂	3	6	19	"	152	235	370	510	455	585	607	



7* ♀	4	6	25	Killed.	1914 May 20	1914 June 6	1914 July 8	1914 Aug. 11	1914 Sept. 4	1914 Oct. 20	1914 Dec. 15	
7C ♀	4	6	25	"	175	180	252	365	302	430	440	
					180	200	220	305	280	380	380	
					1914	1914	1914	1914	1914	1914		
8 ♀	1	6	8	"	June 4	July 8	Aug. 11	Sept. 4	Oct. 14	Dec. 14		
8C ♀	1	6	8	"	85	240	335	290	475	430		
					72	220	310	200	300	375		
					1914	1914	1914	1914	1914	1914		
9* ♀	1	6	4	"	June 18	July 8	Aug. 11	Sept. 4	Oct. 14	Dec. 21		
9C ♀	1	6	4	"	110	160	235	280	405	410		
					115	150	225	190	315	310		
					1915	1915	1915	1915	1915	1915		
10* ♂	1	3	20	"	Jan. 28	Feb. 9	Feb. 22	Mar. 9	Mar. 23	Apr. 6	1915 Apr. 27	1915 May 11
10C ♂	1	3	20	"	75	130	230	220	290	315	355	370
					70	120	220	220	310	325	390	330
					1915	1915	1915	1915	1915	1915	1915	1915
11* ♀	1	3	17	"	Feb. 3	Feb. 9	Feb. 22	Mar. 9	Mar. 23	Apr. 6	Apr. 27	May 11
11C ♀	1	3	17	"	50	100	190	230	270	325	350	440
					70	135	230	280	325	290	310	480
					1915	1915	1915	1915	1915	1915	1915	1915
12* ♀	1	3	14	"	Feb. 6	Feb. 9	Feb. 22	Mar. 9	Mar. 23	Apr. 6	Apr. 27	May 11
12C ♀	1	3	14	"	70	75	190	220	240	290	300	350
					60	75	155	210	270	315	325	370
					103	103	250	300	345	420	575	600
A† ♂		4†		Killed.	120	180	260	370	410	440	485	500
AC ♀				"	68	100	177	225	300	375	385	435
B* ♂	5‡			"	58	97	172	225	280	356	390	455
BC ♂				"								475
												480

\* The operative and control animal were from the same litter.

† From unpublished experiments of Auchincloss and Park.

‡ Born by Cæsarean section.

TABLE II.

*Body Weight and Weight of Organs in Thymectomized and Control Guinea Pigs.*

No. and sex.	Age.	Killed or died.	Body weight.	Weight of gastro-intestinal tract with contents.		Body weight without gastro-intestinal tract and contents.		Weight of organs.								
				gm.	gm.	gm.	gm.	Liver.	Kidneys.	Heart.	Pancreas.	Testes.	Spleen.	Adrenals.	Thyroids.	Thymus.
1* ♀	2 15	Died.	190	46	144	7.5	2.1	0.95	0.65			0.34	0.25	0.01		
1 C ♀	2 15	"	140	38	102	5.8	1.7	0.5	0.57			0.6	0.2	0.05		
2* ♀	2 15	Killed.	265	85	180	13.5	1.4	0.8	1.05			0.32	0.25	0.07		
2 C ♀	2 15	Died.	140	38	102	5.8	1.7	0.5	0.57			0.6	0.25	0.05		
3* ♀	6 20	Killed.	560	141	419	21.5	3.4	1.8	1.6			0.67	0.32	0.1		
3 C ♀	6 20	"	500	113	387	25.3	4.2	2.23	1.7			0.79	0.36	0.05		
4* ♀	7 25	"	520	136	384	38.0	5.52	2.32	1.67			0.92	0.33	0.065		
4 C ♀	7 25	"	530	160	370	48.1	5.0	1.8	1.2			0.92	0.41	0.038		
5* ♀	6 7	"	385	84	301	26.0	3.67	1.58	1.2			0.42	0.33	0.03		
5 C ♀	6 7	"	450	115	335	21.0	3.7	1.9	1.5			0.46	0.21	0.025		
6 ♀	6 19	"	552	126	426	37.5	6.8	1.9	1.77			0.82	0.4	0.1		
6 C ♂	6 19	"	607	127	480	34.6	4.9	2.3	1.89	1.66		0.62	0.28	0.11		0.21
7* ♀	6 25	"	440	134	306	19.5	3.15	1.1	1.0			0.55	0.26	0.01		
7 C ♀	6 25	"	380	119	261	17.0	3.0	1.05	1.15			0.43	0.3	0.03		0.13
8 ♀	6 8	"	430	121	309	26.2	3.7	2.0	1.7			0.7	0.45	0.05		
8 C ♀	6 8	"	375	85	290	24.0	4.0	1.7	1.5			0.6	0.37	0.05		0.35
9* ♀	6 4	"	410	104	306	31.5	4.1	1.48	1.38			0.45	0.35	0.1		
9 C ♀	6 4	"	310	83	227	28.0	3.2	1.45	0.8			0.4	0.35	0.1		0.2
10* ♂	3 20	"	335	95	240	13.7	2.7	1.0	1.05			0.45	0.3	0.01		
10 C ♂	3 20	"	355	97	258	15.5	3.3	0.8	1.2	2.31		0.3	0.3	0.05		0.2
11* ♀	3 17	"	425	87	338	12.7	3.1	1.21	1.21			0.31	0.21	0.08		

11 C ♀	3	17	Killed.	460	101	359	13.4	3.61	1.36	1.05	0.56	0.25	0.06	0.55
12* ♀	3	14	"	310	67	243	12.61	2.81	0.85	1.11	0.35	0.2	0.07	
12 C ♀	3	14	"	335	95	240	13.4	3.11	1.1	1.1	0.36	0.2	0.05	0.4
13† N ♀	3	8	Killed.	410	90	320	10.4	2.55	1.15	1.2	0.5	0.3	0.05	0.2
14 ♂	3	13	"	230	58	172	9.0	2.01	1.0	0.81	1.21	0.15	0.01	
15 N ♂	3	19	"	370	132	238	14.0	3.0	1.05	1.15	2.8	0.31	0.21	0.16
16 ♀	5	10	"	560	105	455	19.8	4.3	1.4	1.3	0.85	0.4	0.1	
17 N ♀	6	14	"	455	94	361	36.5	4.1	1.5	1.6	0.62	0.32	0.12	0.2
18 ♂	7	4	"	475	97	378	13.5	4.01	1.15	1.21	3.8	0.45	0.31	0.1
19 ♀	8	13	"	512	127	385	33.7	4.2	2.13	1.55	0.83	0.4	0.12	
20 ♂	11		"	525	152	373	26.46	4.82	2.17	1.52	2.62	0.62	0.35	0.06
21 ♂	11	9	"	605	126	479	39.4	5.16	2.5	1.25	3.5	0.66	0.55	0.15
22 N ♂	11	22	"	595	131	464	28.5	4.4	2.4	1.8	0.5	0.27	0.04	0.6

\* Both animals were from the same litter.

† This group of six thymectomized and four normal animals contains single survivors of experiments. The thymectomized members of the group have been used for the study of the effects of the removal of the thymus on the organs, and, for this purpose, the normal animals may be regarded as controls.

of 103 gm., amounting in most cases to about one-fourth of the total weight of the animal. If the weights of the gastro-intestinal tracts and actual body weights (body weight minus weight of gastro-intestinal tract) are compared, it will be seen that the living healthy guinea pig, fed in the usual way, is composed of 2 to 5 parts of body and 1 part of food.<sup>4</sup>

No histological changes, which were constant, could be found in any of the organs of internal secretion or in the bones. There were no signs of rickets. The femora and ribs cut before decalcification with the same stony resistance as the bones of the controls, and on microscopical examination showed no osteoid zone bordering the trabeculae or irregularity in the growth of the cartilage. The thyroids displayed marked individual differences, as has been noted in other animals by different observers, but no variation common to one set of guinea pigs. The adrenals presented no constant differences such as have been described by Matti in the dog and also by Klose. Measurements of the cortex and medulla of the adrenals of the pairs of animals showed how variable the thickness of the medulla is in the guinea pig. Where the adrenal is flat and broad, the medulla is scarcely more than a line in thickness; where the adrenal has a more rounded shape, the medulla may have a diameter of 3 mm. The cortex measured uniformly about 1 mm. in thickness. Histologically the adrenals, hypophyses, and ovaries, and the testes of the few males in the experiments, were normal.

Although the negative experiments just described furnish no evidence for or against Paton's conclusion that extirpation of the thymus in immature guinea pigs causes the testes to hypertrophy, yet there are reasons why Paton's work should not be accepted on its face value. His problem concerned growth, and in the investigation of a problem of that nature the cardinal principle is to plan the experiments so as to insure absolute parallelism between each pair of experimental and control animals, and the value of the results obtained depends largely on the strictness with which this principle has been carried out. But Paton chose his controls largely from the same batches of animals and guessed at the ages from the weights, though exact correspondence

<sup>4</sup> The error of estimating the age of the guinea pig from the weight should be considered.

in age between each pair of animals was of vital importance to the success of his experiments. And, finally, he made no allowance for the weight of the gastric and intestinal contents in his determinations of the final weights of his animals. These considerations do not prove Paton's results wrong, but they show that his methods were inaccurate and make corroboration of his work necessary. Halnan and Marshall have repeated Paton's work and failed to corroborate it. The changes which Soli found in the testes of guinea pigs after thymectomy were so slight that his experiments in that animal may be considered negative. In the light of these facts it seems proper to regard the thymectomy experiments on guinea pigs of all investigators up to the present time negative.

Whether the failure to obtain changes in the guinea pig by means of thymectomy indicates that the thymus has no function in that animal gross enough to be brought out in that way, or whether it signifies that the reduction of the thymus to the accessory lobes is insufficient to excite signs of thymus insufficiency in the guinea pig is a question which cannot be answered in the present state of our knowledge; and to speculate in regard to it is idle, until the principle that thymus function can be demonstrated by thymus extirpation has been firmly established in other animals in which complete or more nearly complete removal of the thymus is possible.

#### CONCLUSIONS.

1. Accessory lobes of thymus, derived from the third pharyngeal pouch, occurring in close association with the parathyroids from the third pouch, were found in serial section of the cervical tissues of eleven out of fourteen guinea pigs, and probably would have been found in all fourteen but for a technical error.
2. It is probable, therefore, that accessory lobes of thymus having this situation and origin are usually, if not always, present in the guinea pig.
3. Additional accessory lobes of thymus belonging to, but at some distance from the main lobe were also present in several of the animals.
4. The discovery of these accessory lobes makes it certain that the guinea pig is unsuitable material for complete thymectomy, and prob-



ably complete extirpation of the thymus in this animal is rarely, if ever accomplished.

5. The extirpation experiments of previous investigators in the guinea pig must now be regarded as partial extirpations, and their results interpreted in that light.

6. Extirpation of the thymus in the guinea pig produced no changes in the writer's experiments.

7. The study of the serial sections of the cervical tissues of the guinea pig indicates that Ruben's statements regarding the parathyroid derived from the fourth pharyngeal pouch in the guinea pig are correct,—that it is much smaller than parathyroid III, may be rudimentary, and is sometimes absent at least on one side.

8. No accessory lobe of thymus was found accompanying the parathyroid from the fourth pouch, a finding also bearing out Ruben's statement that no thymus anlage springs from the fourth pouch in the guinea pig.

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## EXPLANATION OF PLATES.

## PLATE 20.

FIG. 1. The right thyroid is shown with the parathyroid derived from the fourth pharyngeal pouch imbedded in its substance, and the parathyroid derived from the third pouch accompanied by an accessory lobe of thymus, lying on its outer border. *a*, accessory lobe of thymus; *b*, parathyroid from the third pouch; *c*, right thyroid; *d*, parathyroid derived from the fourth pharyngeal pouch.

FIG. 2. The parathyroid derived from the third pharyngeal pouch appears as a thin strand, which terminates anteriorly in an accessory lobe of the thymus also derived from the third pouch. The strand of parathyroid tissue measures two and one-half times the length of the accessory thymus lobe; less than half of it is included in the photograph. The thyroid does not appear as it lies too far posteriorly. *a*, small accessory lobe of thymus; *b*, larger accessory lobe; *c*, parathyroid continuous with thymus lobe; *d*, salivary glands; *e*, cartilage of trachea.

## PLATE 21.

FIG. 3. Accessory lobes of thymus are shown clustering around the parathyroid from the third pouch. Two separate masses of parathyroid are shown which, in serial section, could be seen to be continuous. In the larger of the two masses a small island of thymus can be seen. A study of the other sections shows it

not to be an island, but an arm of thymus tissue thrust into the parathyroid. In the smaller of the two masses of the parathyroid there is thymus tissue and also a duct-like space, lined with cylindrical epithelium. *a*, accessory lobes of thymus derived from the third pouch; *b*, parathyroid from the third pouch; *c*, duct-like space lined with cylindrical epithelium.

FIG. 4. The same parathyroid and group of accessory thymus lobes as shown in Fig. 3, but at a different level. *a*, thymus from the third pouch; *b*, parathyroid from the third pouch; *c*, duct-like space lined with cylindrical epithelium.

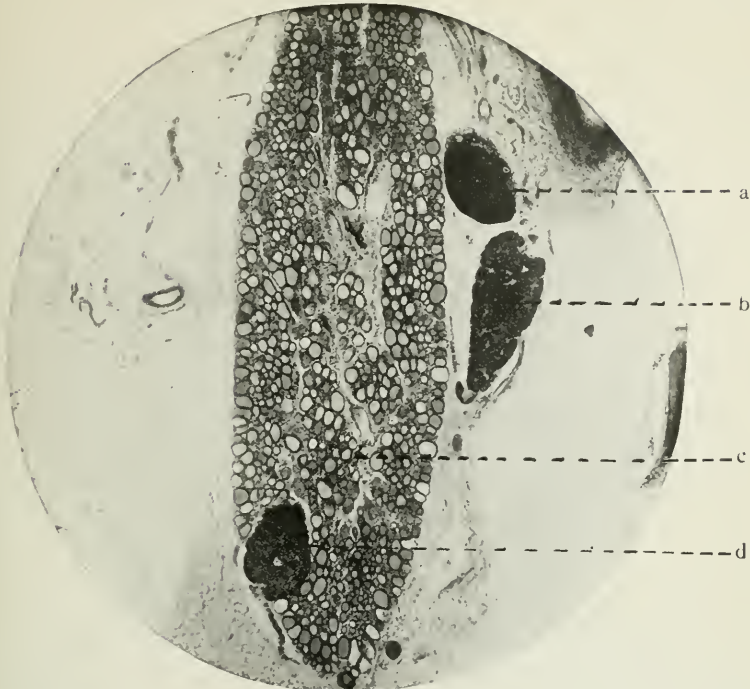


FIG. 1.

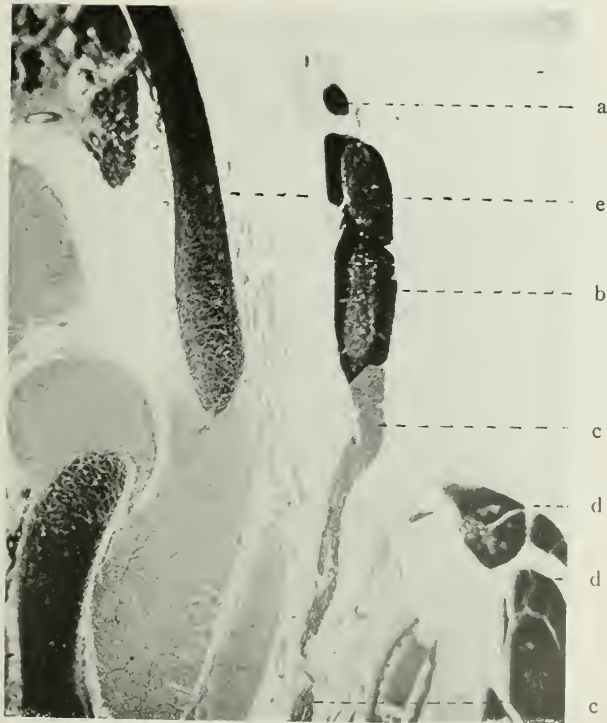


FIG. 2.

(Park: Extirpation of the Thymus in the Guinea Pig.)





FIG. 3.

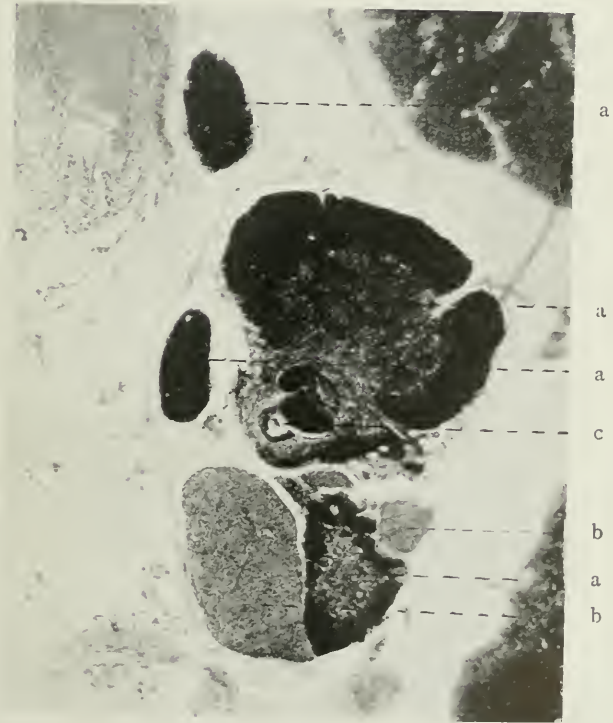


FIG. 4.

(Park: Extirpation of the Thymus in the Guinea Pig.)





# EXPERIMENTAL STUDIES ON THE RELATION OF THE PITUITARY BODY TO RENAL FUNCTION.

By KETIL MOTZFELDT, M.D.

(From the Laboratory of Surgical Research of Harvard Medical School, Boston.)

(Received for publication, September 29, 1916.)

## INTRODUCTION.

In recent papers I have dealt with the clinical aspects of the relation between the pituitary body and the kidneys, especially with regard to the etiology and pathology of diabetes insipidus. I have come to the conclusion that the pituitary body, as shown by its extracts, exerts a constant, physiological influence on the functional activity of the kidneys in human beings. This action consists in a checking of the flow of urine,—an antidiuretic effect which is most marked when the diuresis is high. I have also reported a case of diabetes insipidus in which organotherapy with the posterior lobe of the pituitary body has been successfully carried out for a period of about 2 years.

This field for investigation is a comparatively new one, and the subject is of great importance not only from a physiological point of view, but from the promise it gives of an improved therapy. During the past year I have investigated this question experimentally.

## HISTORICAL.

That there is some connection between the pituitary body and renal activity was first shown in 1901 by Magnus and Schäfer. They found a pronounced increase in the flow of urine in dogs, cats, monkeys, and rats, following the injections of pituitary extracts, and concluded that this influence was exerted directly on the epithelial elements of the kidneys. The same results were obtained by Schäfer and Herring in 1906, though their records show that the influence is far from constant.

Houghton and Merrill, in 1908, found that the increase in flow of urine in the dog lasted on an average of 19 minutes, and that the effect was dependent on the increase in the general blood pressure. A year later Halliburton, Candler,

and Sikes claimed that the extracts caused an increased output in cats, and in 1910 Ott and Scott came to the same conclusion.

Pentimalli and Quercia, in 1912, from experiments on the isolated kidney of the rabbit, showed that hypophysial extracts diminished the flow as well from the ureter as from the renal vein.

King and Stoland, in 1913, working on etherized dogs, were able to demonstrate an increase in the general blood pressure for 10 minutes, accompanied by swelling of the kidney with subsequent temporary increased flow of urine lasting for 20 minutes. They hold the view that the increased flow is due to renal vasodilatation. In the same year Gabriels, working on the isolated kidney of the dog, found increased flow without vasodilatation. On the other hand, Beco and Plumier, in 1913, noted a transient diminution of flow. In the same year Iscovesco maintained that a lipoid from the anterior lobe had a diuretic action both in rabbits and man.

In 1914, Römer reported that the flow was decreased in rabbits and cats, catheterized every hour.

Hoskins and Means, in 1912, concluded that the diuretic effect found in dogs under narcosis was not due to changes in blood pressure.

Herring, in 1913 and 1914, stated that the diuretic effect of the posterior lobe extracts could be demonstrated in all species of animals, and that extracts from the anterior lobe and the pars intermedia did not possess this power.

Cow, in 1914, found that pituitrin had a diuretic action of 15 minutes' duration in cats.

In 1916, von Meyenburg showed that the injection of extracts caused a decreased flow in rabbits of 8 to 10 hours' duration.

Shamoff, in 1916, found that electric stimulation of the superior cervical ganglion produced increased flow due to excitation of the pituitary body, but his results were inconstant.

The results obtained from observations in man are almost as contradictory.

De Cyon, in 1910, mentions that he had seen diuretic effects from feeding dry pituitary preparations.

Falta, Newburgh, and Nobel, in 1911, found increased diuresis a common result from subcutaneous injections of pituitrin.

Von den Velden, in 1913, was able to check the diuresis in two normal men by hypophysin, and in the same year Frey and Kumpiess confirmed this result by injections of pituglandol. 2 years later von Konschegg and Schuster obtained the same result in four normal subjects. Motzfeldt, in 1914, also found that injections of pituglandol checked the diuresis in a series of fifteen relatively healthy patients, while the anterior lobe extracts did not possess this property.

During the past 3 years a number of cases of diabetes insipidus have been reported in which pituitary extracts have checked diuresis to a considerable extent.

As will be seen, there has been great confusion concerning this question, and this is due chiefly to the fact that results of the experiments in the physiological laboratories have been too readily accepted by clinicians. As a matter of fact, every text-book dealing with this matter states as an ascertained fact that extracts from the posterior lobe are diuretics.

At this point it should be emphasized that the term diuresis is used with different meanings. Some authors use the word to indicate the total 24 hour amount of urine, without regard to the quantity, while others define the term as increase of the quantity of urine.

The confusion is due chiefly to the methods employed. Schäfer carried out his original experiments on anesthetized animals with the drop-recording method, and the observation time was generally very short. The urine was collected from a cannula inserted directly into the bladder. Under these circumstances it cannot be excluded that the increased number of drops may be due, at least partly, to the tonic action on the bladder, which is a constant effect of pituitary injections. There are, moreover, serious objections to operations on the bladder or ureter, as reflex action may interfere considerably with the normal conditions. Furthermore, as we do not know the influence of narcosis on the vegetative nervous system, work on anesthetized animals introduces an uncontrollable source of error.

Most of the subsequent observations have been made by Schäfer's original method, with the same errors and the same misinterpretation of the results. The results, however, have been notably inconsistent in the reports of all. Schäfer and Herring, for instance, in 19 experiments on dogs, observed a diuretic effect in 12, and in 7 a diminution of flow. Moreover, most of the work has been carried out with intravenous injections which usually influence the blood pressure considerably.

#### *Method.*

The chief aim in finding a suitable method has been to avoid the sources of error mentioned above, and to work as closely as possible under physiological conditions. The work was started on dogs, but experience soon taught that rabbits were more suitable for this

purpose and the majority of the experiments, therefore, have been carried out on these animals.

Having made sure that the 24 hour amount of urine was diminished with three pituitrin injections given at 8 hour intervals, the half hourly output was followed. The animals were placed on the table on their backs and catheterized every half hour. Catheterization of male rabbits is easily done by a soft rubber catheter, and consequently the experiments were carried out on males exclusively.

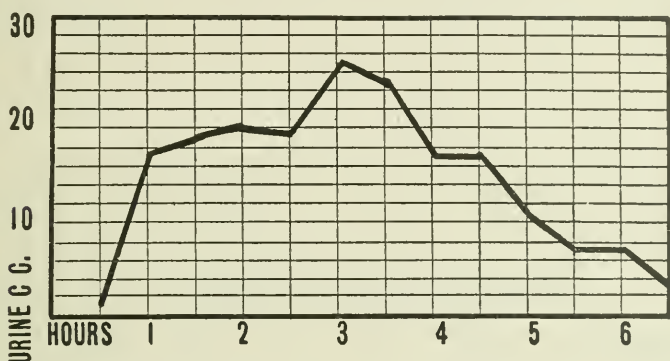
The experience of previous investigators has shown that the position on the back does not interfere with the normal flow of urine and the animals keep perfectly still without anesthesia. Psychic influences, therefore, can be ruled out.

In order to obtain a more marked reaction, I subsequently produced an artificial polyuria. For this purpose 150 cc. and later 200 cc. of water (about 37°C.) were introduced by stomach tube immediately before the animals were placed on the table. This amount is approximately the maximum which the stomach of a medium sized rabbit can hold. This method is much the best way of giving water, the subcutaneous or intravenous administration being less reliable and often unsatisfactory. Wherever the term "artificial polyuria" is used in the legends of the charts, it is to be understood that the polyuria is produced in the way described above.

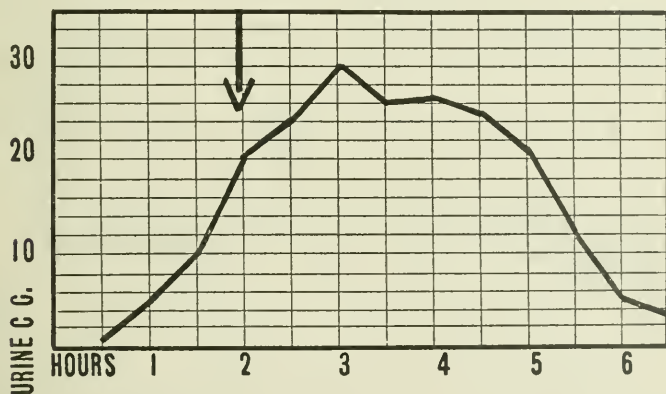
The degree of polyuria is dependent on many factors, particularly, however, on the water content of the tissues, and since this is controlled chiefly by the food, it is of importance to avoid a dry diet and feed the animals mainly on fresh vegetables. Rabbits have occasionally been used several times, as often as every other day, and this is of value because after introduction of water once or twice, the polyuria starts more readily and reaches higher degrees than on the first occasion. The half hourly amounts of urine, obtained in this way, have been plotted as curves, and the resultant polyuria has served as an excellent measure in testing the antidiuretic effect. Only in exceptional instances has the polyuria been delayed for several hours, and, when first started, spontaneous depressions of the output have never been observed.

The polyuria curve is not uniform, and the total amount of urine

returned is subject to great variation. Usually, however, the polyuria starts in 1 to 2 hours and is finished within 4 to 5 hours from the onset (Text-figs. 1 and 2). In estimating the antidiuretic effect, it is, therefore, the relative values which are of chief importance.



TEXT-FIG. 1. Rabbit, weight 2,100 gm. Artificial polyuria. (Water given at the beginning of the observation is indicated by zero.)



TEXT-FIG. 2. Rabbit, weight 1,500 gm. Artificial polyuria. The arrow indicates 0.7 per cent saline solution, 1 cc. intravenously (as control).

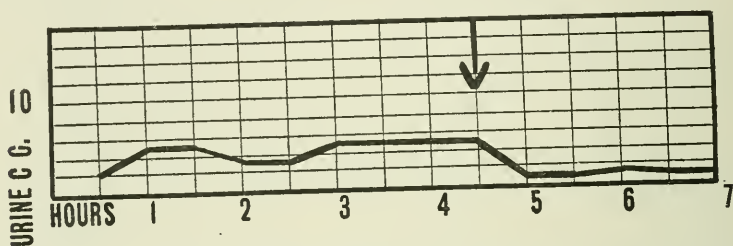
The pituitary extracts employed have been the usual commercial preparations: Pituitrin (Parke, Davis and Company), Pituitary Liquid (Armour and Company), Pituitary Extract (H. K. Mulford Company), Pituglandol (Hoffmann La Roche), Hypophysin (Farbwerke-Hoechst Company), and Extract Hypophysis (Schering and Glatz).



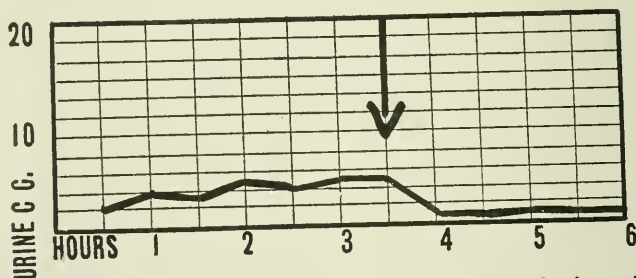
The majority of the experiments have been done without blood pressure registration, and oncometer determinations have not been employed.

*Effect of Pituitary Extracts.*

The normal half hourly output of urine in rabbits is subject to considerable variation and there is no dependable normal. But even under these circumstances the antidiuretic effect (Text-figs. 3 and 4) of hypophyseal extracts is plainly shown (11 observations). It may



TEXT-FIG. 3. Rabbit, weight 1,400 gm. Normal output of urine. The arrow indicates pituitrin, 1 cc. subcutaneously.

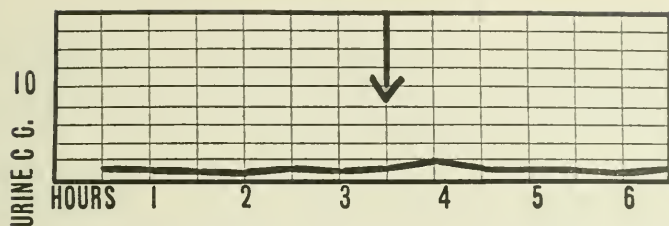


TEXT FIG. 4. Rabbit, weight 1,500 gm. Normal output of urine. The arrow indicates pituitrin, dilution 1: 10, 1 cc. intravenously.

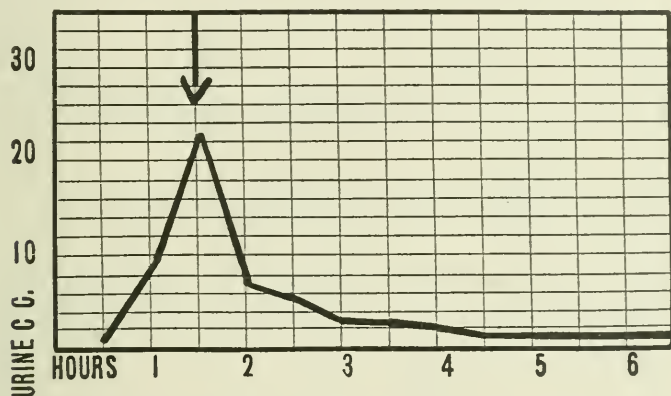
be noted also that the subcutaneous and intravenous injections of the extracts act in the same way, and that when the output is very low (Text-fig. 5) there is no effect to be seen from the injection. This seems to indicate that there is a certain lower limit beyond which the output cannot be depressed. The effect of the extracts on artificial polyuria, however, is far more pronounced (Text-figs. 6 and 7).

Pituitrin diluted 1:50, subcutaneously injected in 1 cc. doses, evidently has power to check the output for approximately 5 to 6

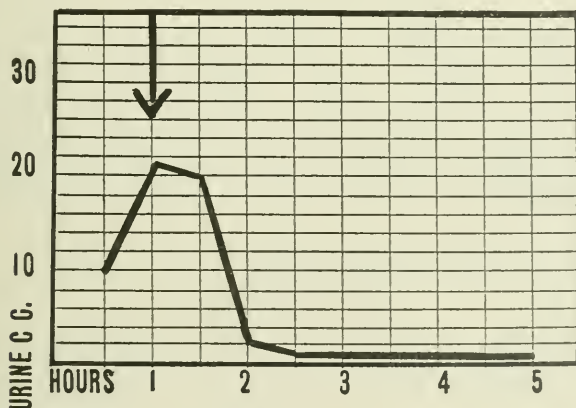




TEXT-FIG. 5. Rabbit, weight 1,200 gm. Normal output of urine. The arrow indicates pituitrin, dilution 1: 10, 1 cc. subcutaneously.

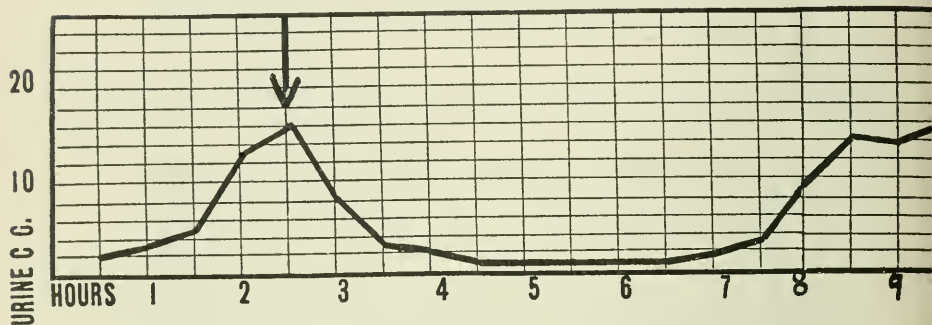


TEXT-FIG. 6. Rabbit, weight 2,000 gm. Artificial polyuria. The arrow indicates pituitary liquid, 1 cc. subcutaneously.

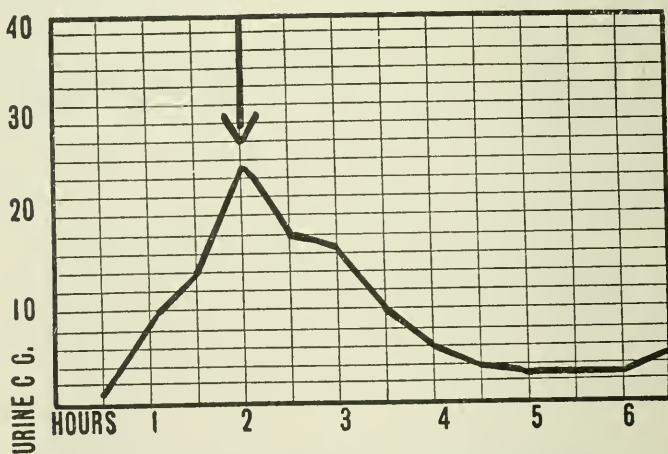


TEXT-FIG. 7. Rabbit, weight 2,400 gm. Artificial polyuria. The arrow indicates pituitary liquid, dilution 1: 10, 1 cc. subcutaneously.

hours (Text-fig. 8). Moreover, it is to be noted (Text-fig. 7) that the influence at times is little marked during the first 30 minutes, and generally reaches its maximum about 2 hours after the injection. This antidiuretic influence may be regarded as constant, for I have



TEXT-FIG. 8. Rabbit, weight 1,600 gm. Artificial polyuria. The arrow indicates pituitrin, dilution 1: 50, 1 cc. subcutaneously.



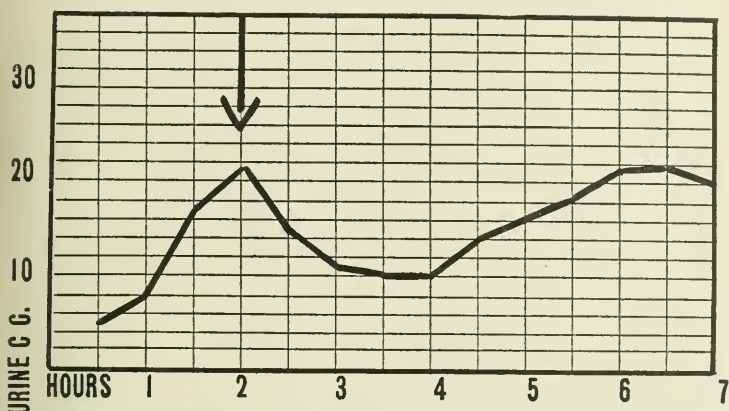
TEXT-FIG. 9. Rabbit, weight 1,400 gm. Artificial polyuria. The arrow indicates pituitrin "400‰," 3 cc. by mouth.

seen no exceptions to this rule. The different pituitary extracts have all acted in the same way.

When the urinary output is depressed, the concentration changes in correspondence with the quantity excreted, from colorless to yellowish brown.

The preparations have not been compared as to limit of activity. Most of the work has been done with pituitrin, and the smallest active dose of this extract depends upon its method of administration.

*Orally.*—(9 observations.) Here there is a slow onset of the renal depression reaching its maximum in about 2 to 3 hours (Text-fig. 9<sup>1</sup>). The lower limit of activity for administration by mouth is apparently 1 cc. of pituitrin (Text-fig. 10).

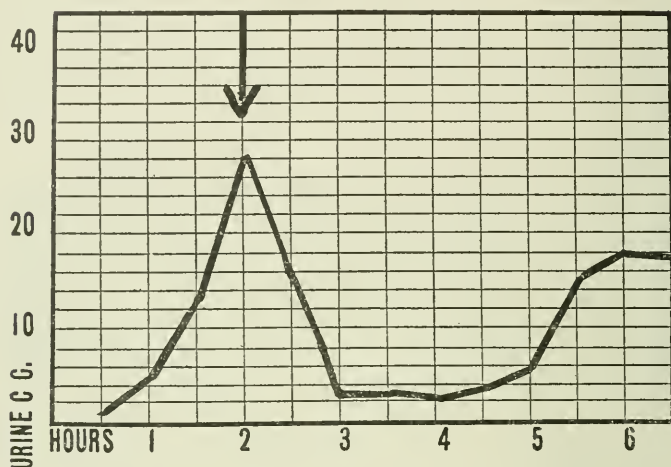


TEXT-FIG. 10. Rabbit, weight 1,500 gm. Artificial polyuria. The arrow indicates pituitrin, 1 cc. by mouth.

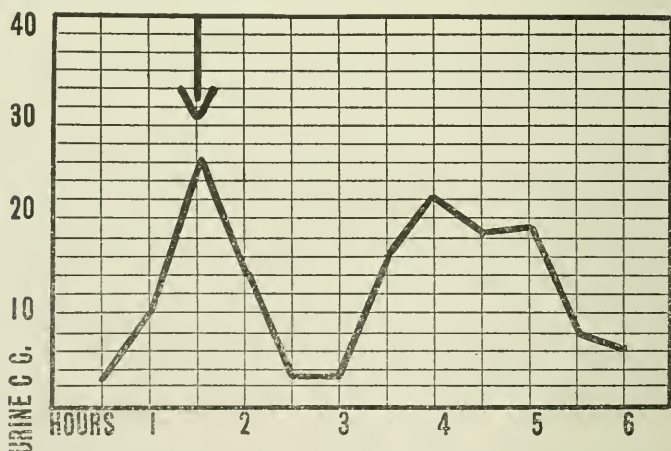
*Subcutaneously.*—(32 observations.) (Text-figs. 11, 12, and 13.) Here the onset is more abrupt, and with decreasing doses the duration of the effect is shorter. In small doses the effect is diminished also to the extent of the depression. These curves seem to indicate a limit of activity in dilution between 1:100,000 and 1:200,000, but the action is not constant beyond a dilution of 1:10,000. How this may be accounted for is unknown. On the other hand, the effect of large doses has been tried with the Parke, Davis preparation "400%." The action sets in rapidly, but even with this enormous dose, the output does not fall below 1 to 2 cc. in half an hour (Text-fig. 14). Apparently it is impossible to produce anuria in this way. The injections were well borne, except for cutaneous necrosis,

<sup>1</sup> Pituitrin "400%" is a special preparation (Parke, Davis and Company), which is four times the normal strength of pituitrin.

probably due to long lasting anemia of the skin, and the animals lived for a long time. Autopsy did not reveal pathological changes.



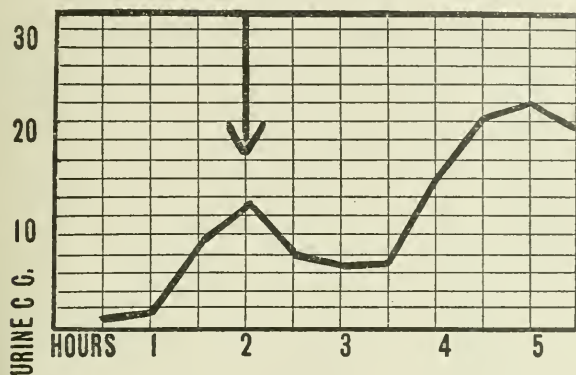
TEXT-FIG. 11. Rabbit, weight 1,600 gm. Artificial polyuria. The arrow indicates pituitrin, dilution 1: 800, 1 cc. subcutaneously.



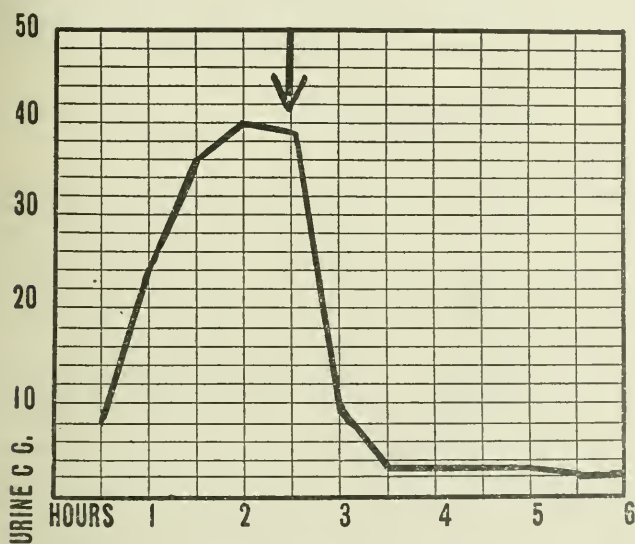
TEXT-FIG. 12. Rabbit, weight 1,800 gm. Artificial polyuria. The arrow indicates pituitrin, dilution 1: 5,000, 1 cc. subcutaneously.

*Intravenously.*—(8 observations.) This mode of administration shows the same result of decreasing doses as to duration and degree

of depression (Text-figs. 15 and 16). Evidently the limit by this method is beyond 1 cc., dilution 1:500,000 (Text-fig. 16), which



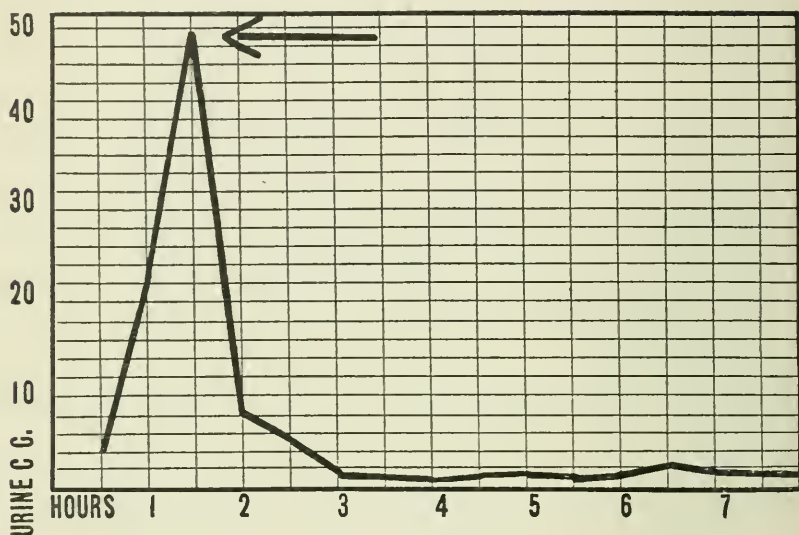
TEXT-FIG. 13. Rabbit, weight 2,200 gm. Artificial polyuria. The arrow indicates pituitrin, dilution 1:100,000, 1 cc. subcutaneously.



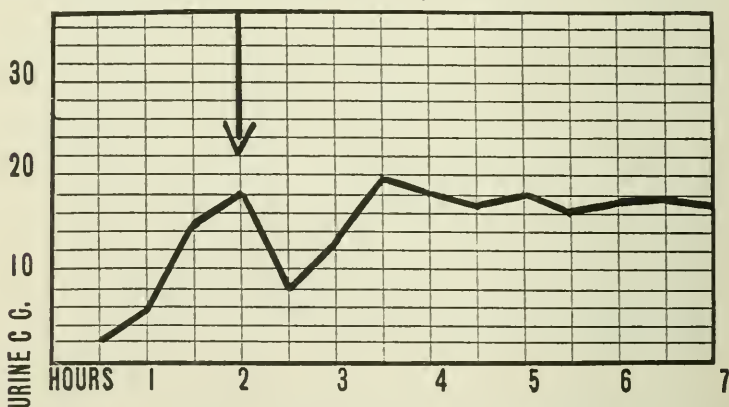
TEXT FIG. 14. Rabbit, weight 2,100 gm. Artificial polyuria. The arrow indicates pituitrin "400%," 12 cc. subcutaneously.

might possibly be of value as a biological test for the presence of pituitary secretion. My observations, however, are too few to warrant conclusions as to its reliability for this purpose. The effect

sets in more quickly, but in some instances there has been a latent period even by intravenous injections.



TEXT-FIG. 15. Rabbit, weight 2,800 gm. Artificial polyuria. The arrow indicates pituitrin, 2 cc. intravenously.



TEXT-FIG. 16. Rabbit, weight 1,500 gm. Artificial polyuria. The arrow indicates pituitrin, dilution 1: 500,000, 1 cc. intravenously.

As already mentioned, dogs are not suitable for experiments of this kind on account of the high concentration of their urine. I have,



however, made two observations on a dog in which polyuria had developed after partial pancreatectomy.<sup>2</sup>

*Observation 1.*—Dog; weight 13.2 kg. October, 1915. Partial pancreatectomy. Lowered carbohydrate tolerance. Sugar-free on meat diet. Diuresis averaged 200 to 300 cc. April, 1916, diuresis averaged 500 to 700 cc. (no sugar).

*Pituitrin 1 Cc. Five Times Subcutaneously.*

		<i>Diuresis.</i>	
		cc.	Sp. gr.
Apr. 27		600	1,028
" 28		460	1,025
" 29		500	1,032
" 30		620	1,035
May 1		600	1,040
" 2		650	1,028
" 3		750	1,032
" 4		400	1,034
" 5		200	1,047
" 6		700	1,030

*Observation 2.*—The same dog.

*Pituitrin 1 Cc. Three Times Subcutaneously.*

		<i>Diuresis.</i>	
		cc.	Sp. gr.
May 9		850	1,015
" 10		900	1,013
" 11		250	1,047
" 12		600	1,030
" 13		770	1,017
" 14		600	1,016
" 15		520	1,028

*Is This Effect Dependent on Changes in General Blood Pressure?*

*A priori* this seems unlikely, as it is agreed by most investigators that a subcutaneous injection of pituitrin exerts a slight influence or none on the blood pressure, and it would require a pronounced drop to account for the antidiuretic effect. Three observations in which the pressure has been registered for 3 hours after subcutaneous injection with pituitrin, have shown no change in pressure, while the output has been checked.

<sup>2</sup> I am indebted to Dr. J. Homans for the opportunity of making these observations.

*To Which Part of the Pituitary Body Is This Action Due?*

Watery extracts have been prepared from different parts of the same lot of bovine glands, made up in the same way as is pituitrin.

Anterior lobe extract (5 observations) gave a definite antidiuretic reaction in a dose of 1 cc., while 1 cc. of a dilution of 1:50 gave negative results.

The extracts from the pars intermedia (7 observations) and the posterior lobe (10 observations), however, had a marked antidiuretic influence in a dilution of 1:40,000 (1 cc. injected). The intermedia extract showed this effect most constantly, while the posterior lobe extract failed in some instances long before the dilution 1:40,000 was reached. The dilutions were not carried beyond 1:40,000, and subcutaneous injections were used exclusively.

Two other extracts from the anterior lobe (Parke, Davis and Company) also showed negative results when 1 cc. of a dilution of 1:50 was injected. These anterior lobe extracts have also produced a marked response on the guinea pig uterus (Dale apparatus).

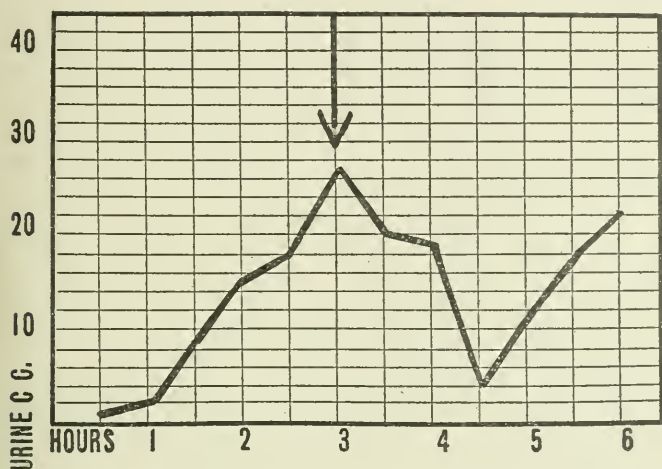
A special anterior lobe preparation, tethelin, which Robertson claims to be the growth-controlling principle of that lobe, has further been tested (4 observations). 1 cc. of a 5 per cent solution of this substance had a marked antidiuretic effect, and the limit of activity is between dilutions of 1:10 and 1:100.

It would thus appear justifiable to assume that the anterior lobe is relatively weak in antidiuretic action, while this power seems to be equally manifested by the pars intermedia and by the posterior lobe. These results are in agreement with clinical experience in as far as anterior lobe extracts are unable to check the output in diabetes insipidus.

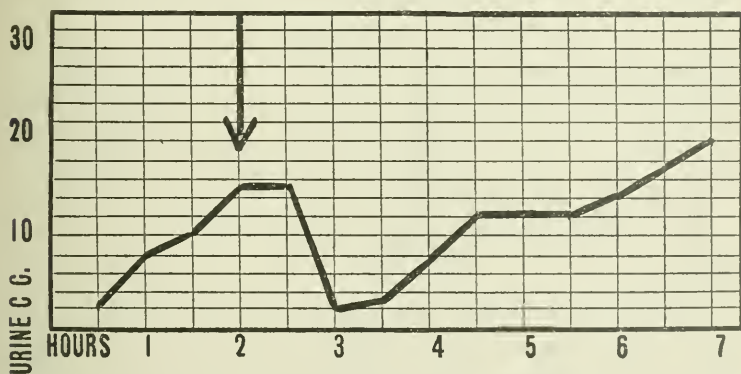
*Active Principles of the Hypophysis.*

In spite of the large amount of work done upon this subject, especially in the chemical laboratories of some of the leading drug firms, our knowledge is comparatively limited. Many compounds of doubtful specificity have been isolated, but although the term "active principle" is not yet justifiable, some of the bases isolated may be regarded at least in some way as functional equivalents. One of these compounds,  $\beta$ -imidazolethylamine, is already used for the standardization of pituitary extracts.

Owing to the European war it has been very difficult to obtain these materials, and I have been able to secure small samples only of some of the compounds (Hoffmann La Roche).



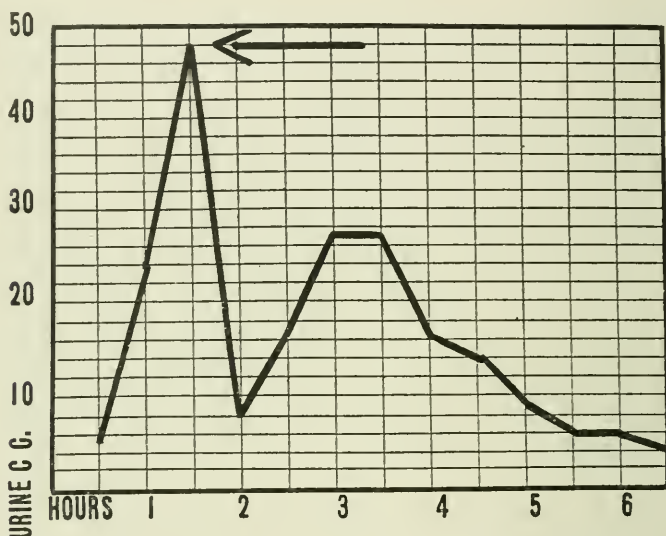
TEXT-FIG. 17. Rabbit, weight 1,500 gm. Artificial polyuria. The arrow indicates  $\beta$ -imidazolelethylamine (Hoffmann La Roche), 20 mg. by mouth.



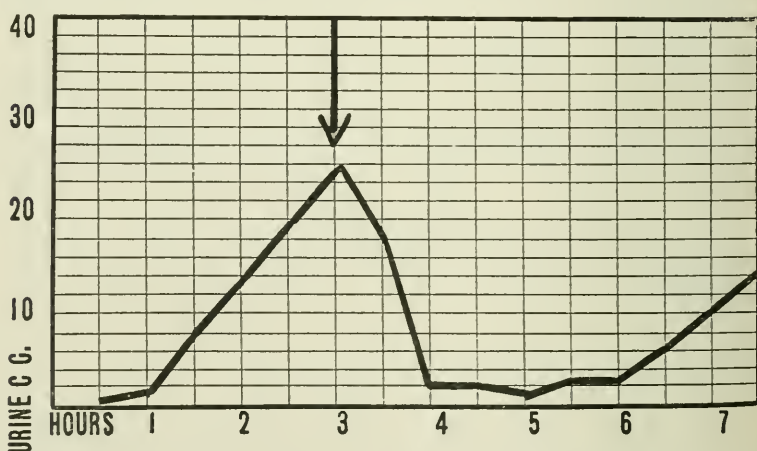
TEXT-FIG. 18. Rabbit, weight 1,700 gm. Artificial polyuria. The arrow indicates  $\beta$ -imidazolelethylamine, 1 mg. subcutaneously.

*$\beta$ -Imidazolelethylamine. Orally.*—(5 observations.) For 20 mg., the curves (Text-fig. 17) are in perfect agreement with the pituitary curves. 10 mg. had no such effect, and when exposed to air and light for 24 hours the solution had become inactive.

*Subcutaneously.*—(6 observations.) The curve for 1 mg. showed a positive result (Text-fig. 18), while 0.4 mg. only gave a slight anti-diuretic response.



TEXT-FIG. 19. Rabbit, weight 2,000 gm. Artificial polyuria. The arrow indicates  $\beta$ -imidazolethylamine, 1.5 mg. intravenously.

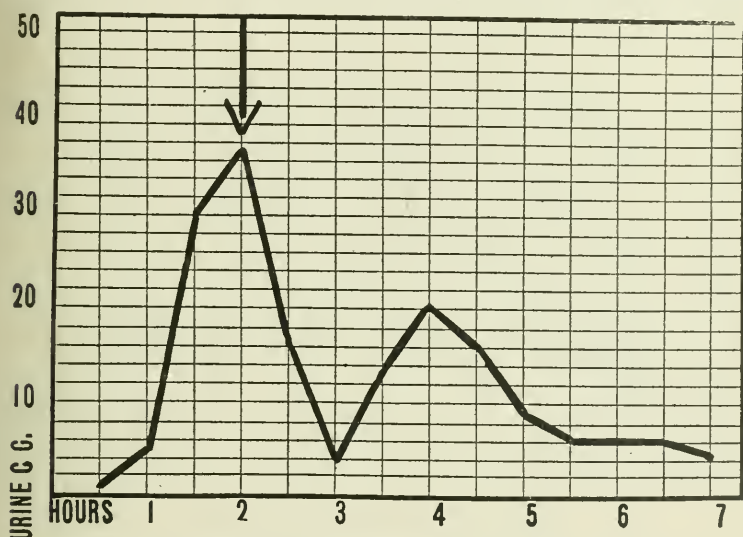


TEXT-FIG. 20. Rabbit, weight 1,500 gm. Artificial polyuria. The arrow indicates *p*-oxyphenylethylamine (Hoffmann La Roche), 10 mg. subcutaneously.

*Intravenously*.—(2 observations.) Strongly positive results were obtained for 1.5 mg. (Text-fig. 19).

*p-Oxyphenylethylamine*.—(1 observation.) Strongly positive results were obtained with 10 mg. subcutaneously injected (Text-fig. 20).

Experiments with the mother substances of these amines, histidine (1 observation) and phenylethylamine (1 observation), in 10 mg. doses, gave negative results. In this connection it will be of interest to see the effect of *Secale* (5 observations), the drug from which Barger and Dale first isolated  $\beta$ -imidazolyethylamine. A prepara-



TEXT-FIG. 21. Rabbit, weight 2,000 gm. Artificial polyuria. The arrow indicates secacornin (Hoffmann La Roche), dilution 1: 10, 1 cc. subcutaneously.

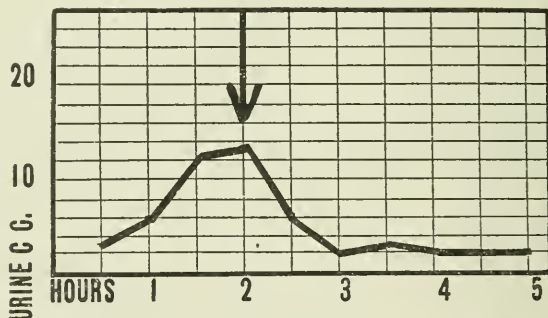
tion from this drug gave positive results in a 1: 10 dilution (Text-fig. 21), while the reaction was almost negative in a dilution of 1: 50. This finding will naturally imply that the sympathetic nervous system or the blood vessels were involved in the action. Moreover, it is of interest because *Secale* is one of the drugs recommended in diabetes insipidus.

These results are suggestive, as the compounds belong to the series of amines which Barger and Dale have named "sympathomimetic



amines" on account of their general stimulating effect on the sympathetic nervous system. Unfortunately, it is at present impossible to procure in America other members of this highly interesting chemical group. Their antidiuretic action has not previously been demonstrated, but Dale and Laidlaw observed a decrease in the volume of the kidney following injection with  $\beta$ -imidazolyethylamine, which they believed due to active renal vasoconstriction. They found no effect on the secretion of urine,—probably on account of the short observation time.

Barbour and Quagliariello have been able to show that  $\beta$ -imidazolyethylamine constricts isolated arteries.



TEXT-FIG. 22. Rabbit, weight 1,500 gm. 0.7 per cent sodium chloride solution, 200 cc. subcutaneously. The arrow indicates pituitrin, 1 cc. subcutaneously.

#### *Parenteral Water Administration.*

In order to rule out the remote possibility that the antidiuretic action might be due to influence on the intestinal absorption, some experiments have been performed in which polyuria was produced by saline solution, administered subcutaneously (3 observations). The pituitary extracts acted in the usual way (Text-fig. 22).

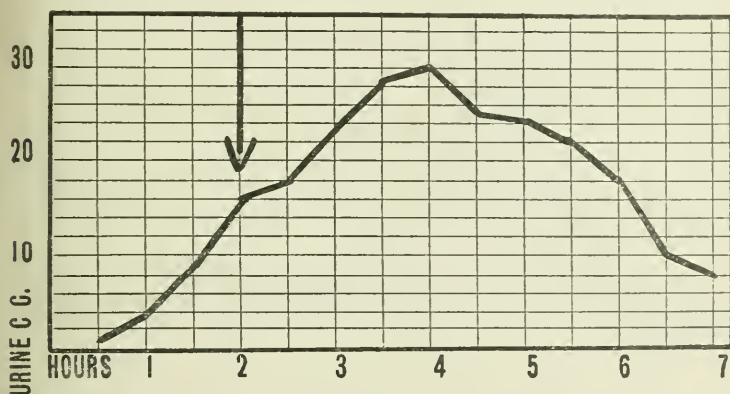
#### *Influence of the Nervous System.*

The marked influence of the nervous system upon the kidneys has been considered chiefly of vasomotor nature, the existence of true secretory nerves being as yet unproved.



*Vagi*.—(10 observations.) Opinions have differed considerably as to the influence of the vagi. Increase or decrease in output of urine has been described as the result both of stimulation and of division of the nerve. Some investigators, however, have been unable to demonstrate any influence.

The vagi were severed in the neck before beginning the experiments. This technique, however, proved unsatisfactory as the onset of polyuria was either delayed or did not occur, and I have reason to believe that this was due to gastric paralysis whereby the ingested water was not moved on. It was necessary, therefore, to postpone the division of the nerves until the water had left the stomach and poly-



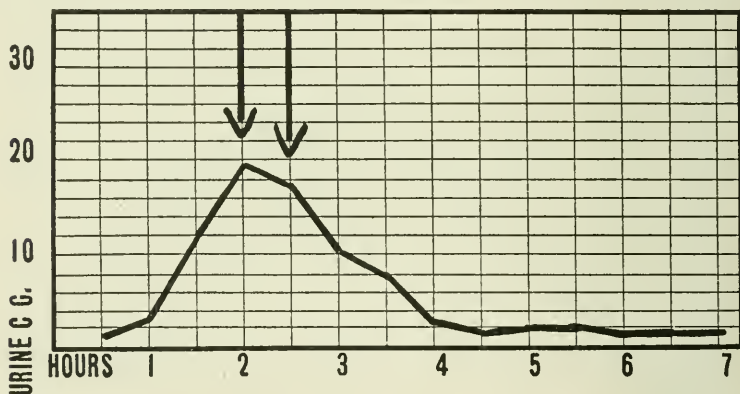
TEXT-FIG. 23. Rabbit, weight 2,000 gm. Artificial polyuria. The arrow indicates the division of the nervi vagi in the neck.

uria had set in. The operations were finished in 5 to 10 minutes and performed under ether narcosis. This procedure (Text-fig. 23) does not interfere with the normal course of the artificial polyuria, and gives a good test-object. The output, however, is checked in the usual way by pituitary injections (Text-fig. 24).

*Splanchnic Nerves*.—(8 observations.) Most investigators agree that stimulation tends to diminish the flow of urine. Division of the nerve is supposed to bring about polyuria in dogs but not in rabbits, though Jungmann and Meyer have observed polyuria of short duration also in these animals. The influence of the splanchnics is generally supposed to be dependent on vasomotor changes.

Section of both splanchnic nerves did not suit my purpose, as polyuria was not readily produced. This may be accounted for partly by the low blood pressure, which is apt to persist after the operation, and partly by the tendency of the animals to develop diarrhea upon water ingestion. Consequently only one splanchnic was cut and the kidney of the other side removed, this procedure being considered safe as several earlier investigators agree that the activity exerted by the splanchnic nerve on the kidney is strictly unilateral.

Splanchnicotomy in rabbits is a difficult operation and this undoubtedly accounts for the varying results. The nerve was exposed

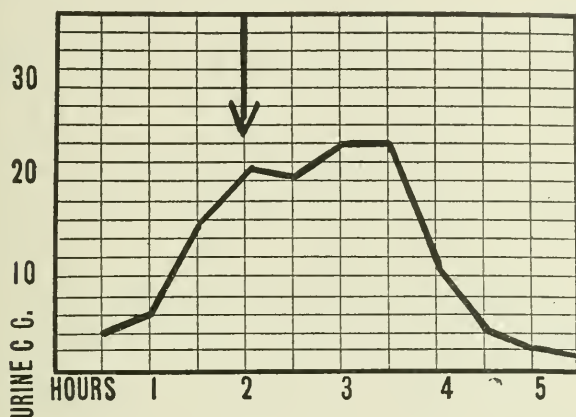


TEXT-FIG. 24. Rabbit, weight 1,400 gm. Artificial polyuria. The first arrow indicates the division of the nervi vagi. The second arrow indicates pituitary liquid, 1 cc. subcutaneously.

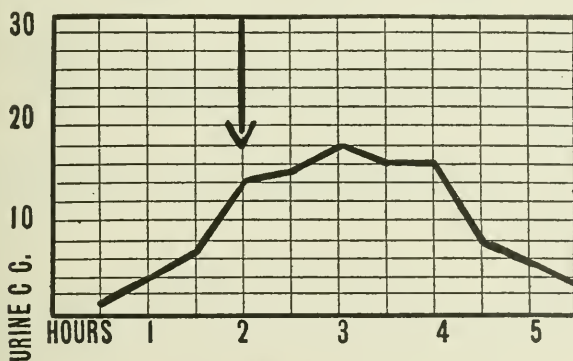
from the back in the retroperitoneal space and divided about 1 cm. above the adrenal, the procedure being performed under ether anesthesia. The nerve is small and the field narrow, and it is difficult, therefore, to be certain that all fibers have been cut. The observations were made 1 to 8 days after operation.

Some of the observations showed the usual response upon pituitary injection, but in others apparently no influence could be demonstrated (Text-figs. 25 and 26). Since, in my experience, 1 cc. of pituitary extract has never failed to check polyuria, I believe it justifiable, despite the limited number of experiments, to infer that the splanchnic nerve is the pathway for the normal antidiuretic action of

the pituitary extracts. If the curves do not justify the conclusion that the influence is entirely absent, it must at least be granted that the reaction is considerably delayed.



TEXT-FIG. 25. Rabbit, weight 1,600 gm. Division of the left splanchnic nerve and removal of the right kidney, 5 days before the observation. Artificial polyuria. The arrow indicates pituitrin, 1 cc. subcutaneously.

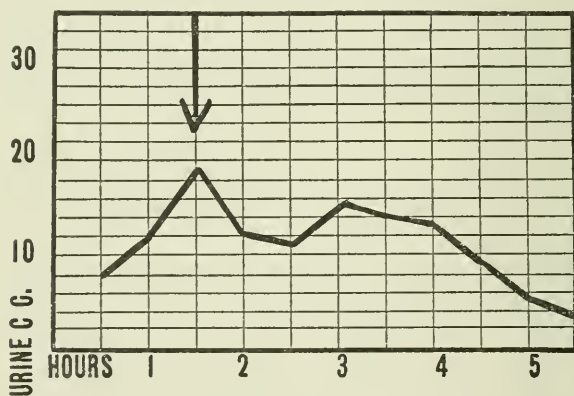


TEXT-FIG. 26. Rabbit, weight 2,000 gm. Division of the left splanchnic nerve and removal of the right kidney, 1 day before the observation. Artificial polyuria. The arrow indicates pituitrin, 1 cc. subcutaneously.

*Renal Nerves.*—(6 observations.) The kidneys of the rabbits were exposed transperitoneally, under anesthesia, through a median abdominal incision and the vessels carefully dissected near the hilus; the visible nerves were cut, and the outer layer of the artery partly

removed. As nerve fibers must still remain in the wall of the artery, it is impossible in this way to exclude nervous influence entirely, but approximately 90 per cent of the fibers are supposed to be divided. The observations were made 2 to 5 days after operation. Some of the animals gave the usual response to pituitrin while others acted in an unusual manner, the differences being possibly referable to variation in the number of functionally intact nerve fibers. As regards both degree and duration, some of the experiments show that the reaction is less pronounced than usual (Text-fig. 27).

The investigations of von Frankl-Hochwart and Fröhlich indicate that pituitrin acts as a stimulus to certain nerves belonging to the



TEXT-FIG. 27. Rabbit, weight 2,400 gm. Division of the renal nerves, 8 days before the observation. Artificial polyuria. The arrow indicates pituitrin. 1 cc. subcutaneously.

sympathetic as well as to the parasympathetic system, while the influence, according to Dale, is exerted directly on the involuntary muscles. As to the kidneys, however, this point has not been tested.

#### *Is the Antidiuretic Influence Caused by Vasomotor Changes?*

The nervous system has, as has been shown, a definite relation to this effect of the pituitary extracts, and the influence may be of vasomotor or secretory origin. As the existence of true secretory renal nerves remains doubtful, a foundation for discussion of this question is lacking. The general vasoconstrictor influence of pituitary ex-

tracts is, on the other hand, well known, but as to the renal vessels there is much confusion, apparently due to the methods employed.

Schäfer and his coworkers noted dilatation of the renal vessels, and Campbell, working on isolated renal vessels, confirmed this finding, while Dale found constriction of the isolated kidney. Pentimalli and Quercia, in perfusion experiments, observed decreased flow from the renal vein. Pal was able to demonstrate that strips from the outer layer relaxed, while the central parts contracted.

In dealing with these problems emphasis must be placed on the existence of peripheral vasomotor centers, the existence of which, in renal vessels, may be regarded as well established. It is, therefore, not justifiable to rule out nervous influence even after section of the nerves. It is possible, furthermore, that antidiuretic influence may be produced while the pituitary extracts are, at the same time, working on the nervous system and directly on the smooth muscles of the vessels.

The vasoconstriction, which I believe is the chief cause of the checked output of urine, may be theoretically induced by stimulation of the vasoconstrictor or paralysis of the vasodilator nerves, but the assumption of an active vasoconstriction is the most likely explanation. With this assumption, the short antidiuretic response after dissection of the renal nerves may be accounted for by the fact that a part of the renal nerves between aorta and hilus is intact, and also by the remaining fibers in the wall of the vessels. Clinically, diabetes insipidus has for many years been considered to be due to vasomotor disturbances.

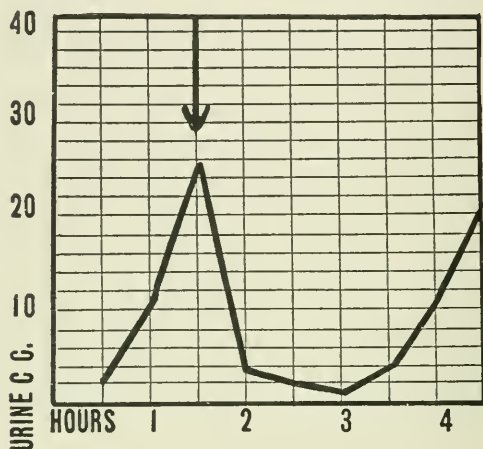
#### *Pharmacological Studies.*

*Nicotine.*—(21 observations.) The influence of this drug shows a close similarity to the effect of the pituitary extracts (Text-fig. 28). The lower limit for the antidiuretic action of nicotine, which influence has not previously been demonstrated, is between 0.1 and 0.05 mg. intravenously injected into rabbits of medium size (Text-fig. 29). Subcutaneous injections with doses up to 30 mg. did not show such an effect. Two observations with blood pressure registration did not show changes in pressure during the time the output was checked.

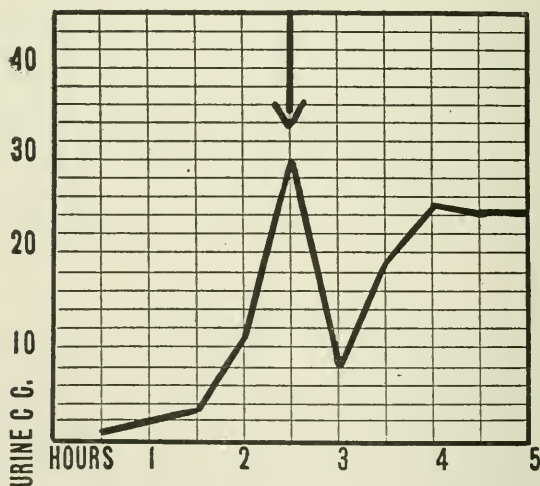
Since the work of Langley and Dickinson, there has been a general



agreement that small intravenous doses of nicotine stimulate the sympathetic nervous system, and it therefore appears justifiable to



TEXT-FIG. 28. Rabbit, weight 2,900 gm. Artificial polyuria. The arrow indicates nicotine, 5 mg. intravenously.



TEXT-FIG. 29. Rabbit, weight 1,500 gm. Artificial polyuria. The arrow indicates nicotine, 0.1 mg. intravenously.

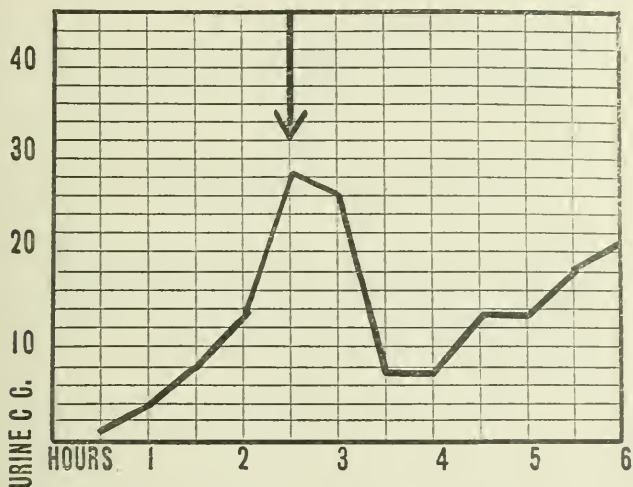
refer the antidiuretic effect to such a stimulation. Judging, furthermore, from Langley and Dickinson's observation that nicotine,



painted on the renal plexus, produced constriction with subsequent dilatation of the renal vessels, it would seem that the antidiuretic power is exercised chiefly by means of these vessels.

I have not been able to block the sympathetic nervous system of the rabbit, as this requires large doses, 7 to 10 mg. per kilo intravenously being given as the smallest effective dose, and these are close to the fatal doses.

*Caffeine.*—(5 observations.) Subcutaneous injections with 0.15 gm. did not influence polyuria, while 0.20 and 0.25 gm. produced an inhibition of only 2 to 3 hours' duration (Text-fig. 30). This is prob-



TEXT-FIG. 30. Rabbit, weight 1,700 gm. Artificial polyuria. The arrow indicates caffeine, 0.25 gm. subcutaneously.

ably due to general vascular constriction, including the renal arteries, although toxic influence with lowered blood pressure could not be eliminated.

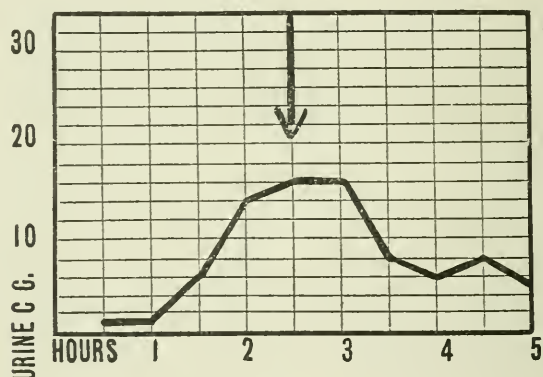
*Strychnine.*—(10 observations.) Subcutaneous injections with sulphate of strychnine, often in convulsive doses, from 0.06 to 0.54 mg., did not inhibit polyuria in rabbits weighing 1.5 to 2 kilos.

Strychnine is among the drugs usually recommended in diabetes insipidus.

*Morphine.*—(4 observations.) Subcutaneous injections with sul-

phate of morphine (8 to 65 mg. in rabbits weighing 1,500 to 1,900 gm.) did not show definite influence on the polyuria curves.

*Chloral and Paraldehyde.*—A series of experiments have been performed in which simultaneously with water ingestion chloral (10 observations) or paraldehyde (6 observations) has been administered orally. The doses have been chosen in accordance with what is given in the text-books as sufficient to paralyze the vasomotor nerves,—0.6 gm. of chloral per kilo, or 3 gm. of paraldehyde for a medium sized rabbit. These are the doses which are necessary to show the diuretic effect of caffeine, when permitted to act for 2 hours.



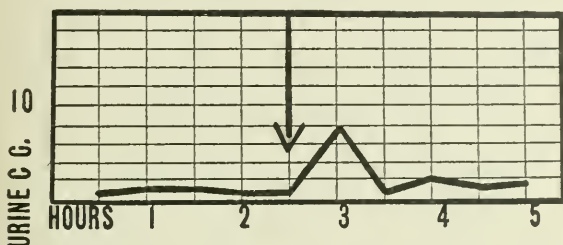
TEXT-FIG. 31. Rabbit, weight 1,300 gm. Artificial polyuria. Chloral 0.8 gm. by mouth. The arrow indicates pituitrin, 1 cc. subcutaneously.

I have been able to confirm the findings of earlier investigators that water diuresis is less readily produced under anesthesia, which fact is probably due to the low blood pressure. There are, however, serious objections against this method of blocking the vasomotor effect. The antidiuretic influence was very little changed under these circumstances (Text-fig. 31), except that the output in some instances did not go so low as after injection in normal animals. These observations disclosed another interesting fact; namely, that an inconstant initial diuretic effect occurred during the first 30 minutes (Text-fig. 32). This is no doubt the effect which has been found by Schäfer and most of the investigators who have worked with his method on anesthetized animals. This view may further be sup-

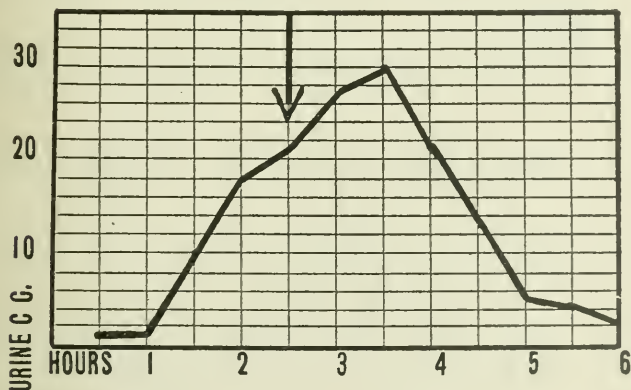
ported by Cow's general experience that when experimenting on unanesthetized animals the diuretic action of a substance may frequently be masked.

*Influence of Other Ductless Glands.*

*Adrenal Medulla.*—(28 observations.) Opinions have differed concerning the influence of adrenalin (Parke, Davis and Company)



TEXT-FIG. 32. Rabbit, weight 1,500 gm. Water, 20 cc. Chloral 0.9 gm. by mouth. The arrow indicates pituitrin, 1 cc. subcutaneously.

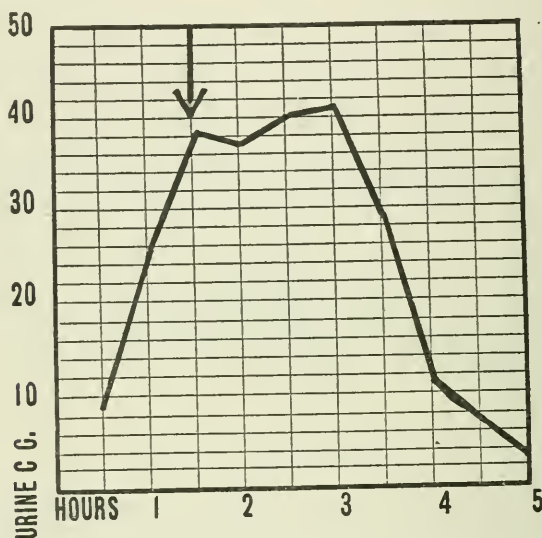


TEXT-FIG. 33. Rabbit, weight 1,800 gm. Artificial polyuria. The arrow indicates adrenalin, 2 mg. subcutaneously.

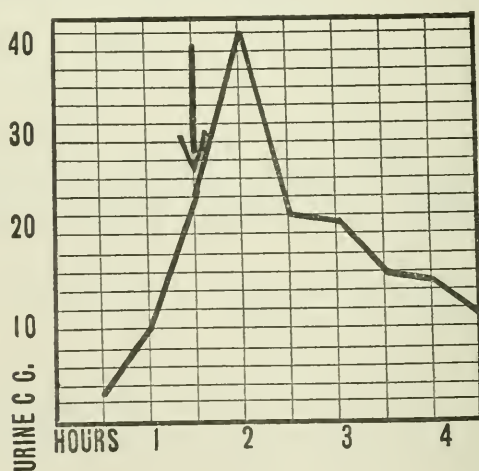
on diuresis. Most writers, however, agree that the general effect is a short, primary decrease and secondary increase in flow of urine. Judging from the knowledge that adrenalin has a constricting action also on the renal vessels and that pituitary extracts stimulate the adrenals to increased secretion, it would *a priori* appear reasonable to assume a cooperation between these glands as to antidiuretic effect.

*Subcutaneously.*—The polyuria is uninfluenced (Text-fig. 33).

*Intravenously.*—The appearance of the polyuria curve is not changed (Text-fig. 34).

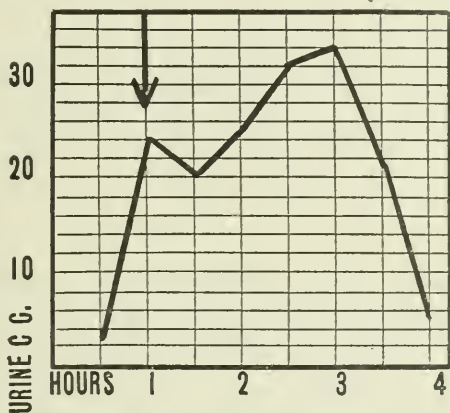


TEXT-FIG. 34. Rabbit, weight 1,700 gm. Artificial polyuria. The arrow indicates adrenalin, 0.01 gm. intravenously.



TEXT-FIG. 35. Rabbit, weight 3,000 gm. Artificial polyuria. The arrow indicates adrenalin, 0.05 mg. intravenously, every 3rd minute for 30 minutes.

*Intravenously at Short Intervals.*—As the effect of an intravenous injection with adrenalin is only of short duration, the drug has in some instances been administered intravenously every 3rd minute for about half an hour. In some instances there seems to be an increase in the output (Text-fig. 35), while other observations have shown a slight decrease (Text-fig. 36). An inhibition, however, corresponding to the effect of pituitary extracts has not been observed. Doses ranging from 0.001 to 2 mg. have been injected, and many of the animals have shown the usual signs of adrenalin intoxication at autopsy.



TEXT-FIG. 36. Rabbit, weight 2,900 gm. Artificial polyuria. The arrow indicates adrenalin, 0.05 mg. intravenously, every 3rd minute for 24 minutes.

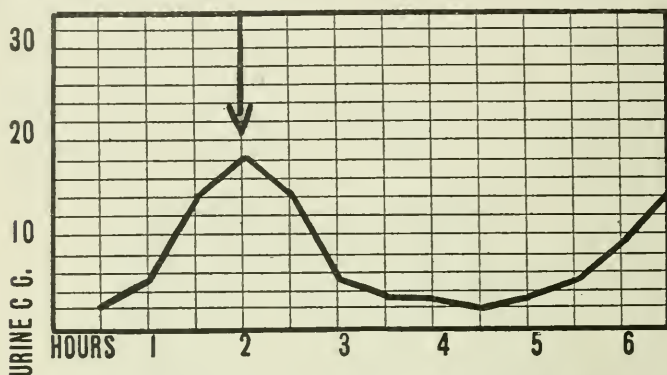
In order to try the influence of other ductless glands, watery extracts have been prepared (Parke, Davis and Company) from different glands, made up in the same way as is pituitrin.

*Adrenal Cortex.*—(11 observations.) This showed a marked anti-diuretic response, when administered subcutaneously in 1 and 2 cc. doses (Text-fig. 37), but the result was negative in a dilution of 1:10, and extract containing medulla as well as cortex did not possess this power in 1 cc. doses. The antidiuretic action of the adrenal cortex is in full harmony with the therapeutic results which Belfield obtained in diabetes insipidus by feeding the cortex. It is not likely, however, that the adrenals are concerned in the etiology of diabetes insipidus, judging from the fact that polyuria is not seen either in Addison's disease or after experimental removal of the adrenals.

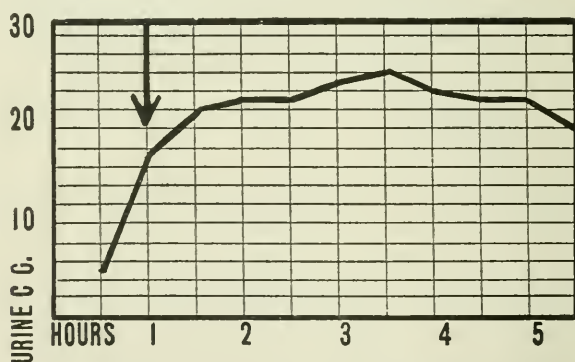


*Pineal Gland.*—(6 observations.) Subcutaneous (Text-fig. 38) and intravenous injections both gave negative results.

There are a few cases of diabetes insipidus on record in which tumors of the pineal gland are held responsible for the polyuria (von



TEXT-FIG. 37. Rabbit, weight 1,800 gm. Artificial polyuria. The arrow indicates adrenal cortex extract, 2 cc. subcutaneously.



TEXT-FIG. 38. Rabbit, weight 1,800 gm. Artificial polyuria. The arrow indicates pineal extract, 1 cc. subcutaneously.

Gierke), but these cases may easily be referred to secondary influence on the pituitary body due to hydrocephalus.

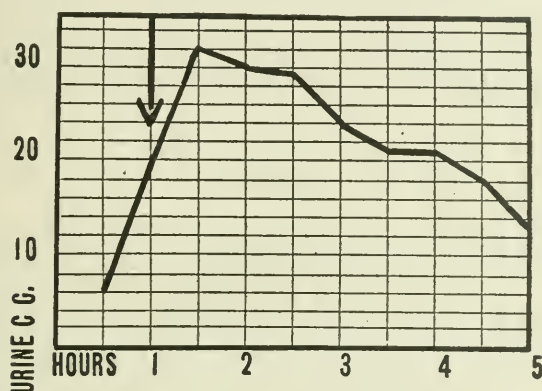
*Thyroid.*—(6 observations.) Both subcutaneous (Text-fig. 39) and intravenous injections were negative.

*Thymus.*—(7 observations.) The results were negative (Text-fig. 40).

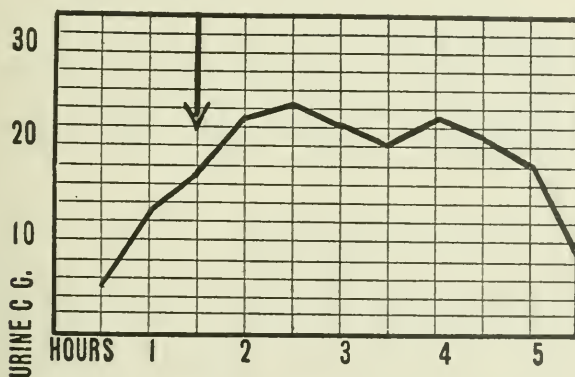


*Corpora lutea*.—(5 observations.) These results were also negative (Text-fig. 41).

*Pancreas*.—(8 observations.) Injections gave negative results (Text-fig. 42) and no explanation is thus found for the polyuria com-



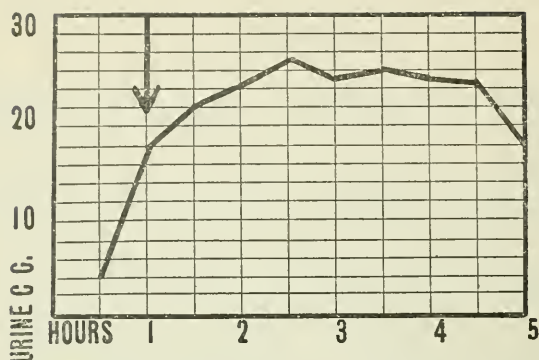
TEXT-FIG. 39. Rabbit, weight 1,900 gm. Artificial polyuria. The arrow indicates thyroid extract, 2 cc. subcutaneously.



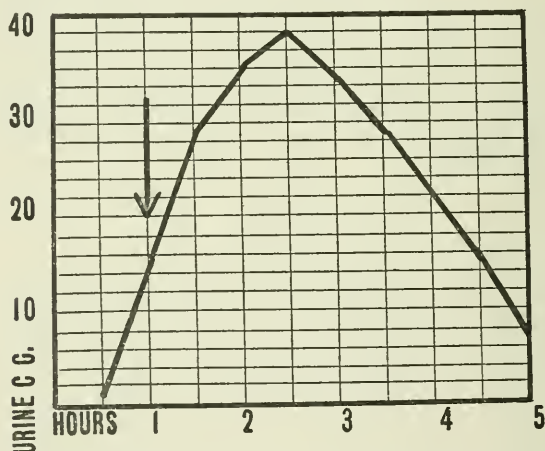
TEXT-FIG. 40. Rabbit, weight 2,600 gm. Artificial polyuria. The arrow indicates thymus extract, 1 cc. intravenously.

monly seen in dogs after partial pancreatectomy, or for the polyuria in diabetic patients.

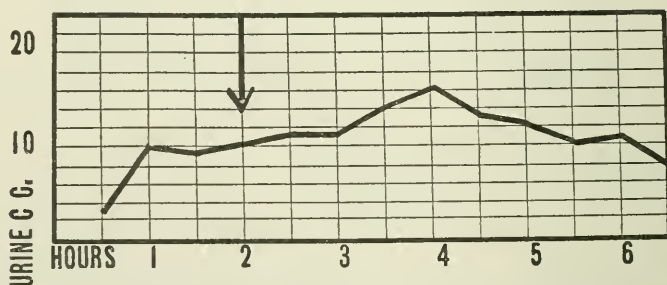
Ott and Scott have seen a diuretic effect from different ductless gland extracts, but the antidiuretic effect has not previously been tested.



TEXT-FIG. 41. Rabbit, weight 1,200 gm. Artificial polyuria. The arrow indicates corpora lutea extract, 1 cc. subcutaneously.



TEXT-FIG. 42. Rabbit, weight 2,100 gm. Artificial polyuria. The arrow indicates pancreas extract, 1 cc. intravenously.



TEXT-FIG. 43. Rabbit, weight 1,700 gm. 10 per cent sodium chloride solution, 40 cc. by mouth. The arrow indicates pituitrin, 1 cc. subcutaneously.

*Influence on the Salt Diuresis.*

Polyuria was produced for this purpose by ingestion by mouth of 40 cc. of a 10 per cent solution of sodium chloride (16 observations). The usual effective dose of pituitrin did not influence polyuria produced in this way (Text-fig. 43), or the influence was only slight. These results tend to demonstrate the fundamental differences between water diuresis and salt diuresis, and they show further the importance of a salt-poor diet in organotherapy of diabetes insipidus.

## SUMMARY.

1. The inconstant results of past observations on the relation of pituitary extracts to renal activity have been due chiefly to unsuitable methods.

2. A standard curve of artificially induced polyuria may be plotted for rabbits, giving 200 cc. of water by mouth.

3. Extracts of the pars intermedia and posterior lobe of the hypophysis, given by mouth, subcutaneously, or intravenously, are able definitely to check polyuria thus induced. Extracts of the anterior lobe show a similar effect, but only to a slight degree.

4. This antidiuretic effect is constant, and is independent of (a) changes in blood pressure, (b) intestinal absorption, and (c) the vagi. The effect is apparently prevented or delayed by division of the splanchnics, and is diminished by division of the renal nerves near the hilus.

5. A similar antidiuretic property is possessed: (a) by  $\beta$ -imidazolyethylamine, (b) by *p*-oxyphenylethylamine, (c) by a preparation from *Secale cornutum*, (d) by small doses of nicotine, (e) by large doses of caffeine, and (f) by extracts of the adrenal cortex.

6. No effect on the polyuria is produced: (a) by strychnine, (b) by morphine, (c) by adrenalin, or by extracts of (d) thyroid, (e) thymus, (f) pineal, (g) pancreas, or (h) corpora lutea.

7. In animals under chloral or paraldehyde anesthesia a short and inconstant initial increase in flow of urine is seen.

8. The antidiuretic effect is absent or only slightly marked in checking the so called salt diuresis.

## CONCLUSIONS.

These facts tend to suggest that the antidiuretic action exerted by pituitary extracts on rabbits is caused by stimulation of the sympathetic nervous system and that the renal vasomotor system in this respect is of chief importance.

Clinically these conceptions bring the polyurias related to disorders of the nervous system and the polyurias of pituitary origin in closer contact.

I wish to acknowledge the assistance of Dr. S. C. Harvey and Dr. W. C. Quinby in some of the operative procedures, the cooperation of Dr. J. Homans and Dr. T. Brailsford Robertson, and the courtesy of Hoffmann La Roche Company. I am indebted to Dr. Harvey Cushing for the privilege of carrying out this work in his laboratory under his direction.

I also wish to express special thanks to Dr. E. M. Houghton and Dr. C. P. McCord, of the biological laboratory of Parke, Davis and Company, for their help.

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# THE PHAGOCYTIC POWER OF CONNECTIVE TISSUE CELLS.

By F. S. JONES, V.M.D., AND PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 22 AND 23.

(Received for publication, October 25, 1916.)

Suspensions of individual, living cells from the fixed tissues can be obtained by digesting with trypsin the clot of proliferating tissue cultures.<sup>1</sup> Certain of the cells thus freed, especially those of connective tissue and the sarcomata, will survive in Locke's solution for many hours, and proliferate when reimplanted in plasma. The method has made possible direct tests of the phagocytic power of fibroblasts.

In connective tissue *in vivo* phagocytosis of bacteria or particles of unorganized matter occurs not infrequently. It can sometimes be seen about bacterial foci and it is common in the neighborhood of old blood extravasations. Lambert and Hanes<sup>2</sup> have shown that carmine particles are ingested by some of the cells of *in vitro* cultures of sarcomatous tissue. But we need scarcely point out that in even the simplest of these instances the tissue is a congeries of cells of at least two sorts; namely, fibroblasts and endothelial cells from the blood and lymph channels. The latter are known to have phagocytic power. It is possible that their activity, with that of wandering cells, may account for the positive findings cited.

## Method.

For our experiments bits of the heart and skeletal muscle from embryo chicks in the 3rd week of incubation, from embryo rats near to term, and from rats 2 to 5 days old were implanted in plasma of the appropriate species. Special care was taken to rule out the

<sup>1</sup> Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 549.

<sup>2</sup> Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiii, 495.

presence of blood, since some of the white cells might, by their phagocytic activity, have introduced confusion into the results. The tissue was washed free of blood by perfusing the animal with Locke's solution<sup>3</sup> injected into the heart, and the effectiveness of the washing, as well as the normality of the tissue, was controlled histologically. The plasma was centrifugalized at high speed, and the central portion drawn off for use through a fine pipette. The microscope showed it to be cell-free.

The cultures were made in small Petri dishes. Sometimes the undiluted plasma was used, and at other times it was mixed with from one to three parts of Locke's solution. After 24 to 48 hours' incubation of the cultures they were submitted to trypsin, and the freed cells were separated from the tissue fragments by filtration through gauze, were twice washed with Locke's solution by means of the centrifuge, and suspended in this fluid. 24 hour cultures yielded the best results. After longer periods the cells were apt to contain fat droplets.

In cultures made as described the muscle did not proliferate but there was an abundant outgrowth of connective tissue which, on digestion of the clot, furnished a thick suspension of living cells. The ability of these to take up particles of carmine was tested as follows: Finely ground carmine was added to the cell suspension, incubation carried on for 1 or for 2 hours, and the preparations were examined in the fresh, and in fixed films stained with methylene blue. Or the carmine was mixed directly with the plasma medium of the original culture, and when growth had taken place the cells were liberated with trypsin and examined for phagocytosis.

Experiments involving the phagocytosis of bacteria proved more troublesome, owing to difficulty in staining the preparations so that the presence of intracellular organisms and the character of the cells should both be brought out. A Gram-staining organism, *Staphylococcus pyogenes albus*, finally was selected, and the tissue cells were counter-stained with lithium carmine. This gave excellent results. Suspensions of freed cells were employed for the tests. With the cells of chicken connective tissue an antistaphylococcus serum was used,

<sup>3</sup> Locke's modification of Ringer's solution, but without sugar.

derived from chickens injected with cultures of the organism, and with rat cells, rat serum obtained in the same way. Leukocytes of the appropriate species were employed to determine the mixture of cell suspension, bacterial suspension, and serum, optimum for phagocytosis. In all the experiments control preparations without serum were made. Locke's solution was used throughout as a diluent. Incubation was for 2 hours, after which the cells were thrown down with the centrifuge, suspended in a little serum, and films taken.

The tests were many times repeated. The results were the same in all, irrespective of the species furnishing the cells, and whether they came from embryos or new-born animals. Both carmine particles and bacteria were phagocyted (Fig. 1), but always to a very small extent. Even in the best preparations it was necessary to go over many cells in order to find one phagocyte. The result cannot be attributed to unfavorable conditions in the mixtures, such for example as insufficient concentration of cells or bacteria, for the conditions were varied through a wide range, and in control preparations with leukocytes phagocytosis was profuse. Nor can it be laid to injury and death of the cells during the tryptic digestion and the subsequent incubation. Such treatment failed to lessen noticeably the phagocytic activity of leukocytes. The majority of the connective tissue cells were still alive at the conclusion of the tests, as shown by the refusal of the nuclei to stain with trypan blue.<sup>4</sup> Furthermore, as already stated, connective tissue cells freed with trypsin will proliferate when plated anew in plasma. The conclusion seems warranted that our technique was not at fault and that the phagocytic power of the cells derived from proliferating connective tissue is actually slight.

The phagocytosis of bacteria by the connective tissue cells occurred only in the presence of serum.

#### *Nature of the Phagocytes.*

The nature of the phagocytic cells remained to be decided. A study of stained films threw light on this question. In our previous paper<sup>1</sup> mention is made of the striking change in the form of cells which

<sup>4</sup> Rous and Jones, *J. Exp. Med.*, 1916, xxiii, 601.

occurs when they are freed from the clot in which they have been growing. The cells of connective tissue cultures, even those with an attenuated spindle shape, all take an approximately spherical form, and their processes merge in the general cytoplasm. But despite this approach to uniformity two sorts of cells, or rather two cell series, can be distinguished in stained preparations (Figs. 2 and 3). They have been figured in color in the paper referred to. With Wright's stain the cells of the smaller sort, which are much the more numerous, are characterized by a round or oval, pyknotic nucleus and a relatively scanty cytoplasm, while those of the larger kind have an oval, vesicular nucleus and abundant cytoplasm. The cytoplasm of both is somewhat basophilic, staining pale blue, and the smaller cells usually divide before it has reached any considerable bulk. Many of the latter closely resemble the lymphocytes of the blood, although in stained sections of the tissue from which they are derived lymphocytes are not evident. The observation may have a bearing on the long discussed problem of the origin of the cells resembling lymphocytes which appear in granulation tissue.

The differences between the two kinds of cells are striking and are still evident to some extent when a mixed suspension of them is plated out in plasma. The small kind then assume a slender, spindle form with long, and often attenuated processes, and the nuclei become oblong or indeed almost rod-shaped; whereas the large cells remain compact in form, as a rule irregularly stellate, with a few short processes, and the shape of the nucleus does not change. These differences are not always well maintained. The trypan blue test indicates that cells of the large kind die much more quickly when kept in Ringer's solution than do the small ones.

It is possible that here are young and old elements of a single sort; but the morphological evidence is against this and in favor of the assumption that the large cells are endothelial, and the more numerous, smaller ones are fibroblasts. Attempts to demonstrate fibroglia in the latter were unsuccessful, as was to have been expected because of their youth. The large cells, and these only, have in our experiments shown phagocytic activity,—a fact which is in keeping with the known character of endothelial cells.

## SUMMARY.

By the tryptic digestion of cultures *in vitro* of avian and mammalian connective tissue, suspensions of individual, living cells have been obtained. Their ability to phagocyte carmine and bacteria has been tested. The great majority of them fail to take up either, but a few large cells are able to do so. They will ingest bacteria only when serum is present; that is, they require the interaction of opsonins. There is good reason to suppose that the phagocytic cells are endothelial in nature. Should they prove to be fibroblasts, like the other elements present, the fact will remain that the phagocytic power of fibroblasts is practically negligible. Their failure to ingest foreign matter *in vivo* is to be laid not to the obstacles offered by the solidity of the tissue they compose, but to an inherent lack of ability on their part. The phagocytosis of blood pigment, bacteria, etc., which takes place in granulation tissue *in vivo* is probably carried on wholly by endothelial cells and wandering cells.

## EXPLANATION OF PLATES.

## PLATE 22.

FIG. 1. Phagocytosed staphylococci in cells derived from the connective tissue outgrowth from bits of the heart and skeletal muscle of young rats, cultivated *in vitro*. The cells belong to the larger of the two sorts figured in the illustrations which follow. Gram's stain followed by lithium carmine.

FIG. 2. The two sorts of cells liberated from connective tissue cultures by digestion with trypsin. Bits of the heart of a young rat were used for the cultures. Wright's stain.

## PLATE 23.

FIG. 3. The same two sorts of cells but derived this time from cultures of the skeletal muscle of a young rat. In these cultures the intermuscular connective tissue alone had proliferated. Some of the large cells show fat droplets. Wright's stain.





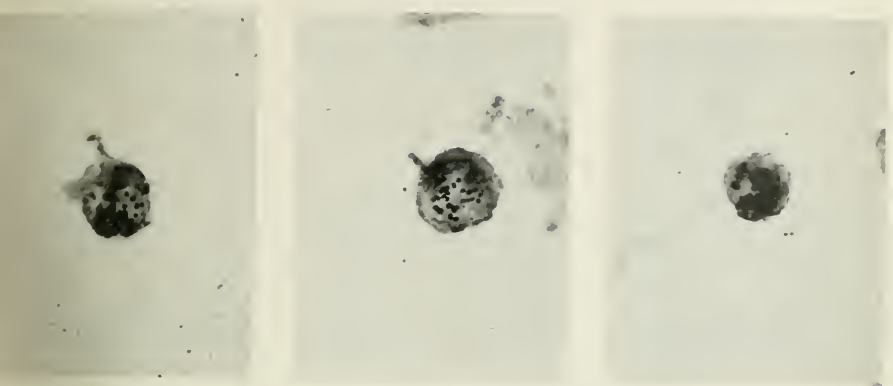


FIG. 1.

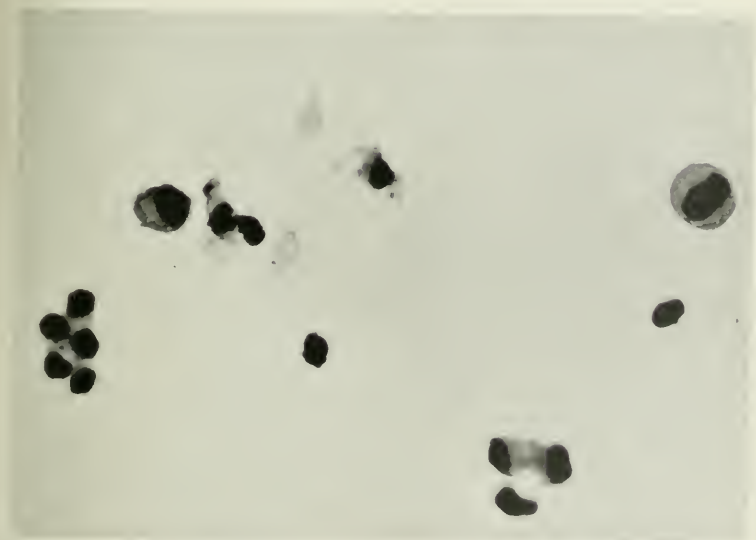


FIG. 2.

(Jones and Rous: Connective Tissue Cells.)



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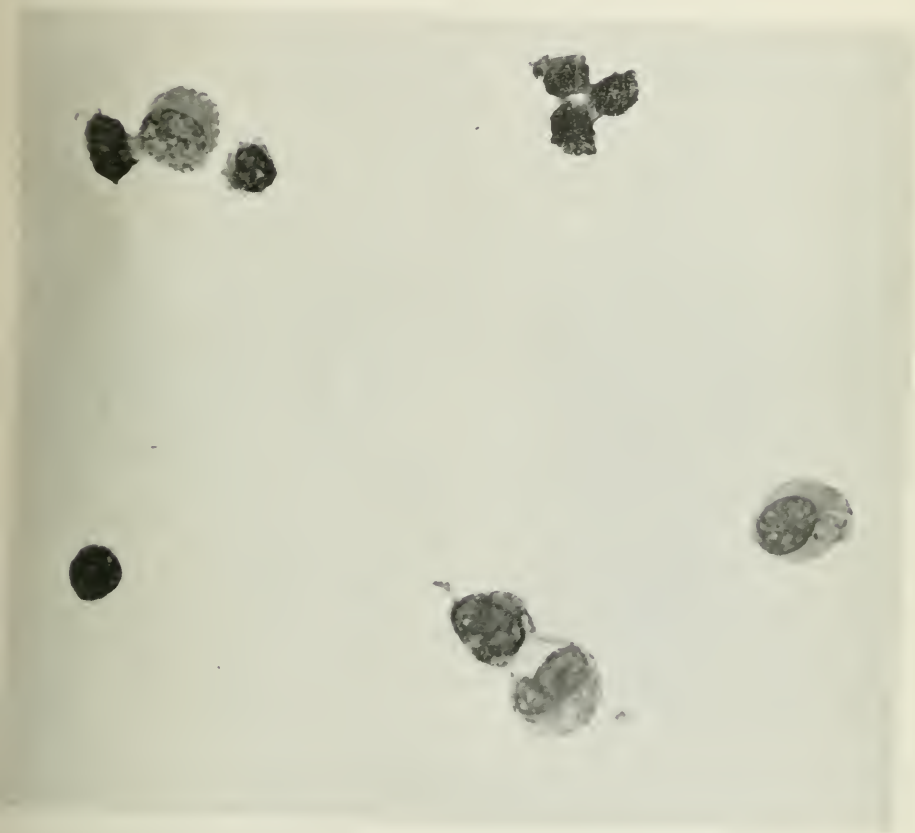


FIG. 3.

(Jones and Rous: Connective Tissue Cells.)



# VENOM HEMOLYSIS AFTER SPLENECTOMY, INCLUDING THE RESISTANCE OF THE ERYTHROCYTES OF NORMAL DOGS TO THE HEMOLYTIC ACTIVITY OF COBRA VENOM.

BY JOHN A. KOLMER, M.D.

(From the McManes Laboratory of Experimental Pathology of the University of Pennsylvania, Philadelphia.)

(Received for publication, September 29, 1916.)

Several investigators have shown that the resistance of erythrocytes to various hemolytic agents is increased after splenectomy. The earlier investigations by Pugliese and Luzzatti (1), Vast (2), Joannovics (3), and Banti (4) were conducted by injecting such hemolytic agents as pyrocin and toluylendiamine into animals before and after splenectomy, and noting in a general manner the increased resistance to hemolysis *in vivo* following the removal of the spleen.

Later investigations by Chaher and Charlet (5), and more particularly by Pearce and his associates (6), have shown the increased resistance of the blood cells to hemolytic agents after splenectomy by experiments *in vivo* and *in vitro*. Chaher and Charlet (5) first drew attention to a slight increase in the resistance of dog erythrocytes to hypotonic salt solutions 10 to 12 days after splenectomy. In a series of researches by Pearce and his associates, Karsner, Austin, Peet, Krumbhaar, and Musser (6, 7, 8, 9), it was shown that the erythrocytes of dogs after splenectomy acquire an increased resistance to hypotonic salt solutions as determined by experiments *in vitro*, and to the influence of specific anti-dog hemolytic serum as shown by experiments *in vivo* and *in vitro*. These investigations have also shown that the increased resistance to hemolysis is due to changes in the erythrocytes themselves.

The relation of splenectomy to the resistance of erythrocytes to the hemolytic activity of cobra venom has not heretofore been investigated. Since the phenomenon of venom hemolysis appears to be intimately associated with the lecithin or ether-soluble activators present in erythrocytes, particularly fatty acids and their soluble soaps, and since the removal of the spleen has been shown to alter the content of fatty substances in the blood, it would appear that an investigation concerning the resistance of erythrocytes to venom before and after splenectomy may yield additional information in this problem.

While the exact nature of venom hemolysis is as yet unknown, the researches in this field indicate that the substance or substances within the erythrocytes acting with the hemolysin in the venom is of a lipoidal nature. Flexner and Noguchi (10), who originally showed that cobra venom is the most active hemolytically of a number of venoms studied, and that the erythrocytes of the dog are susceptible to its influence, believed that the venom hemolysin was activated by serum complement. Later researches by Kyes (11, 12) showed that venom causes the hemolysis of erythrocytes in the absence of serum, and Kyes and Sachs (13) believed that the activating principle was a lecithin contained in the erythrocytes, for which they proposed the name "endocomplement." Noguchi (14), on the other hand, claims that the fatty acids, neutral fats, and soluble soaps in the serum and erythrocytes are the active agents; while other investigators have attempted to correlate these opposing views on the basis that the fatty acids and soaps act as indirect activating agents in venom hemolysis, in that they possess the power of modifying the cell and rendering the intracellular lecithin available for the activation of the venom hemolysin.

The investigations of King (15) indicate that the spleen is concerned in regulating the quantity of unsaturated fatty acids, which are active hemolytic agents, in the blood, and that the benefits derived by splenectomy in pernicious anemia and other diseases, as described by Eppinger (16) and others, is to be ascribed to a reduction of the quantity of these agents in the blood. King has found in experiments conducted with dogs, that the removal of the spleen is followed by a reduction of the unsaturated fatty acids in the serum with an increase of total fats and cholesterol, and that the presence of both the latter and especially of cholesterol, which is well known to possess antihemolytic properties, may account for the increased resistance of the blood to hemolytic agents after splenectomy. However, as shown by Pearce and his associates, the resistance of the blood to hemolytic agents after splenectomy is due to changes in the erythrocytes themselves, which is not explainable on the basis of King's results, especially since King found that the quantity of antihemolytic cholesterol in the corpuscles was decreased after splenectomy, which, it would appear, should render the cells more susceptible to hemolysis, instead of the reverse. Recently Dubin and Pearce (17) reported that analysis, before and after splenectomy, of the blood of dogs shows practically no change in the amount of total fats and unsaturated fatty acids, as expressed by the iodine value.

In the investigation by Kolmer and Pearce (18), on the influence of splenectomy upon the phenomenon of non-specific complement fixation sometimes shown by normal rabbit and dog serum, which has been found by Kolmer (19) to be due largely to lipid substances in the serum, the removal of the spleen did not materially alter the non-specific complement-binding power of the serum, whereas lipid-solving anesthetics, such as chloroform and ether, temporarily removed this property of dog and rabbit sera.



The results of my studies herein reported show that the erythrocytes acquire a temporary increased resistance to venom hemolysis after splenectomy; whether or not this increased resistance is due to a decrease of the lipoidal activator within the cells consequent to the removal of the spleen has not been investigated, but the investigations mentioned show that the phenomenon of venom hemolysis is intimately associated with lipoids and that the spleen may exert an influence upon the lipoidal substances in both corpuscles and serum. Further researches along these lines may explain in definite terms the nature of the increased resistance of erythrocytes after splenectomy, which, at present, is not understood.

### *Method of Study.*

Two separate series of dogs were used in the present investigation; in Series A four animals were splenectomized and two used as controls; in Series B three were splenectomized and two used as controls.

Two or three preliminary tests of the resistance of the washed erythrocytes of each dog to cobra venom were made before the splenectomies were performed, in order to obtain a venom index for each dog. In addition, a large number of normal dogs were examined in order to gain more definite information regarding the normal resistance of the erythrocytes of the dogs with the technique employed.

All the animals were kept on a mixed diet of table scraps, consisting of meat, bread, cereals, and vegetables. As shown by Pearce, Austin, and Pepper (20), the anemia following splenectomy (21) is most marked on this cooked diet as contrasted with a diet of raw meat.

*Venom Tests.*—One lot of cobra venom was used throughout the study.<sup>1</sup> The dried venom was kept tightly stoppered at room temperature and a stock 1:1,000 solution in normal salt solution (0.85 per cent) was freshly prepared each time the venom tests were conducted. From this stock dilution further dilutions with normal salt solution ranging from 1:5,000 to 1:70,000 were prepared.

Twelve dilutions of venom in amounts of 1 cc. each were used

<sup>1</sup> The venom was kindly furnished by Dr. Hideyo Noguchi.

routinely with each blood. To each tube was added 1 cc. of a 4 per cent suspension of washed cells; this doubled the dilutions so that they now ranged from 1:10,000 to 1:140,000. After gentle mixing, the test-tubes were incubated at 37°C. for 2 hours and placed in a refrigerator over night when the readings were made.

In each experiment a hemolytic scale was prepared by dissolving 1 cc. of the mixed cells of four to six dogs in 49 cc. of distilled water. From this 100 per cent solution of 2 per cent of cells further dilutions of hemoglobin were prepared ranging from 5 to 100 per cent. With these the degree of hemolysis in the venom tests were read off and recorded according to the amount of hemoglobin in the supernatant fluid.

Each dog was bled from an external jugular vein through a dry needle in the proportion of 4 cc. of blood in 16 cc. of a 2 per cent solution of sodium citrate in normal salt solution. These suspensions of cells were set aside in the refrigerator over night and washed three times with normal salt solution by low speed centrifugalization. After the last washing a 4 per cent suspension of each lot of cells was prepared in normal salt solution.

Every effort was made to insure uniformity in preparing the dilutions of venom and the suspensions of cells. As venom in dilution tends to deteriorate, it was considered better to prepare a fresh stock dilution for each experiment.

In Series B parallel tests with hypotonic salt solutions were conducted after the method used by Karsner and Pearce (7), except that I used 0.1 cc. of the sediment of washed cells to 3 cc. of each hypotonic salt solution, whereas they used 0.1 cc. of the corpuscular mass secured by centrifuging the blood after defibrination by gentle whipping.

#### RESULTS.

*Venom Hemolysis of the Erythrocytes of Normal Dogs.*—The results of venom tests with the blood of normal dogs and the degree of maximal and minimal resistance are shown in Table I.

As shown in Table I, the resistance of dog erythrocytes to venom hemolysin varies, and the blood of the same dog may vary to a slight extent at different periods. Apparently there is a relation

TABLE I.

*Venom Hemolysis of the Washed Erythrocytes of Normal Dogs (1 Cc. of a Four Per Cent Suspension of Cells + 1 Cc. of Venom Solution).*

No.	Dilution of venom and degree of hemolysis.											
	1:10,000	1:16,000	1:20,000	1:32,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:100,000	1:120,000	1:140,000
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	100	100	100	55	70	50	5	—	—	—	—	—
2	100	100	100	30	60	45	10	8	8	—	—	—
3	100	100	100	60	50	40	18	20	20	15	—	—
4	100	100	100	60	50	45	10	5	5	—	—	—
5	100	100	100	95	90	80	20	10	10	5	—	—
6	100	100	100	80	25	10	5	5	—	—	—	—
7	100	100	100	90	15	10	—	—	—	—	—	—
8	100	100	100	100	100	90	10	10	10	—	—	—
9	100	100	50	5	—	—	—	—	—	—	—	—
10	100	100	100	100	90	60	25	25	25	8	—	—
11	100	100	100	100	100	100	40	40	40	5	—	—
12	100	100	100	90	15	10	—	—	—	—	—	—
13	100	100	100	100	100	100	80	80	20	10	—	—
14	100	100	100	90	20	15	—	—	—	—	—	—
15	100	100	100	90	90	20	—	—	—	—	—	—
16	100	100	100	60	60	60	30	30	—	—	—	—
17	100	100	100	20	—	—	—	—	—	—	—	—
18	100	100	100	20	10	5	—	—	—	—	—	—
19	100	100	100	90	20	15	—	—	—	—	—	—
20	100	100	100	100	90	40	—	—	—	—	—	—
21	100	100	100	80	30	—	—	—	—	—	—	—
22	100	100	100	100	50	5	—	—	—	—	—	—
23	100	100	100	100	30	5	—	—	—	—	—	—
24	100	100	100	100	90	40	—	—	—	—	—	—
25	100	100	100	100	80	60	30	20	—	—	—	—
26	100	100	100	100	100	100	80	60	50	10	—	—
27	100	100	100	90	70	30	10	5	—	—	—	—
28	100	100	100	100	90	60	30	10	—	—	—	—
29	100	100	100	100	100	90	80	40	10	—	—	—

between the degree of anemia which an animal may have due to intestinal parasitism, distemper, or other causes, and the resistance to venom, as the cells become more susceptible to venom in the presence of an anemia. This is important in interpreting the results

of resistance tests after splenectomy on account of the temporary anemia following the removal of the spleen.

On the basis of the examination of the dogs listed in Table I, the maximal resistance of washed dog erythrocytes to cobra venom in our tests may be stated as follows:

Dilution of venom	1: 10,000	gave complete hemolysis in	100 per cent.
" " "	1: 16,000	" " "	100 " "
" " "	1: 20,000	" " "	97 " "
" " "	1: 32,000	" " "	41 " "
" " "	1: 40,000	" " "	17 " "
" " "	1: 50,000	" " "	10 " "
" " "	1: 60,000	does not produce complete hemolysis.	

In general terms, the erythrocytes of the majority of the dogs were not able to withstand an exposure to venom lower than 1: 20,000 before complete hemolysis resulted; hemolysis may begin in dilutions as high as 1: 100,000 of venom, and the point of minimal resistance is more variable, or at least more difficult to read than the point of maximal resistance.

*Venom Hemolysis of the Erythrocytes of Splenectomized Dogs.*—In Tables II and III are shown the results of venom tests with the blood of two of the six dogs belonging to Series A; Text-figs. 1 to 6 were plotted according to the point of maximal resistance, or the

TABLE II.

*Venom Hemolysis of the Erythrocytes of Dog 4 before and after Splenectomy.*

Date.	History.	Dilution of venom and degree of hemolysis.											
		1: 10,000	1: 16,000	1: 20,000	1: 32,000	1: 40,000	1: 50,000	1: 60,000	1: 70,000	1: 80,000	1: 100,000	1: 120,000	1: 140,000
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Oct. 19	1st preliminary.....	100	100	100	90	90	40	5	—	—	—	—	—
" 29	2nd "	100	100	100	60	50	45	10	5	5	—	—	—
Nov. 4	3rd "	100	100	100	50	40	20	10	—	—	—	—	—
" 10	5 days after splenectomy...	100	90	25	—	—	—	—	—	—	—	—	—
" 17	12 " " "	100	50	30	5	—	—	—	—	—	—	—	—
Dec. 7	32 " " "	100	100	100	100	90	20	10	5	—	—	—	—
" 15	40 " " "	100	100	100	100	90	80	80	60	—	—	—	—

TABLE III.

*Venom Hemolysis of the Erythrocytes of Dog 2 Used as a Control.*

Date.	History.	Dilution of venom and degree of hemolysis.											
		1:10,000	1:16,000	1:20,000	1:32,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:100,000	1:120,000	1:140,000
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Oct. 19	1st examination.	100	100	100	100	90	80	60	20	10	—	—	—
" 29	2nd "	100	100	100	30	60	45	10	8	8	—	—	—
Nov. 4	3rd "	100	100	100	90	90	80	60	30	20	10	—	—
" 10	4th "	100	100	100	80	70	60	30	20	20	—	—	—
" 17	5th "	100	100	100	60	40	35	10	—	—	—	—	—
Dec. 7	6th "	100	100	100	100	80	40	10	5	—	—	—	—
" 15	7th "	100	100	100	100	60	50	5	—	—	—	—	—

highest dilution of venom causing complete hemolysis in the various tests. In this series venom tests were conducted 5, 12, 32, and 40 days after splenectomy.

In Series B venom tests were conducted at intervals varying from 4 days to 4 months after splenectomy; in Table IV are shown

TABLE IV.

*Venom Hemolysis of the Erythrocytes of Dog 25 before and after Splenectomy.*

Date.	History.	Dilution of venom and degree of hemolysis.											
		1:10,000	1:16,000	1:20,000	1:32,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:100,000	1:120,000	1:140,000
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Feb. 1	1st preliminary.	100	100	100	100	80	60	30	10	—	—	—	—
" 8	2nd "	100	100	100	90	40	20	—	—	—	—	—	—
" 14	4 days after splenectomy.	100	90	30	10	—	—	—	—	—	—	—	—
" 23	13 " " "	100	95	80	60	30	10	—	—	—	—	—	—
" 29	19 " " "	100	100	90	80	40	5	—	—	—	—	—	—
Mar. 14	33 " " "	100	100	100	90	40	10	—	—	—	—	—	—
" 22	6 wks. " "	100	100	100	100	90	30	10	5	—	—	—	—
Apr. 20	10 " " "	100	100	100	100	100	80	40	10	—	—	—	—
May 25	4 mos. " "	100	100	100	100	100	80	60	10	—	—	—	—

the results observed with one dog of this series, and a summary of the results is shown in Text-figs. 7 to 11, according to the point of maximal resistance, or the highest dilution of venom in each test causing complete hemolysis of the cells.

At the time of the venom tests parallel tests with hypotonic salt solutions were conducted for the purpose of a comparative study. Solutions of sodium chloride ranging from 0.3 to 0.575 per cent were used in each experiment; Table V shows the results observed with one dog of this series. In Text-figs. 7 to 11 I have included a summary of the experiments; the percentage of salt solution given being the point of maximal resistance, or the highest percentage of salt causing just complete hemolysis.

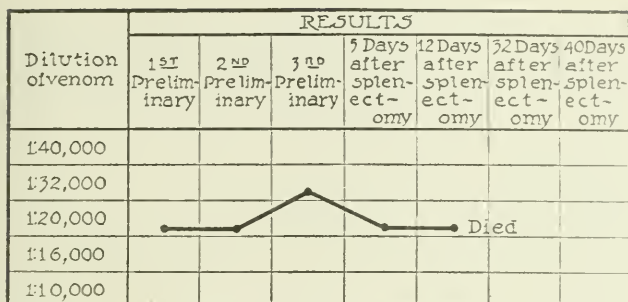
TABLE V.

*Resistance of the Erythrocytes of Dog 25 before and after Splenectomy, to Hypotonic Salt Solutions.*

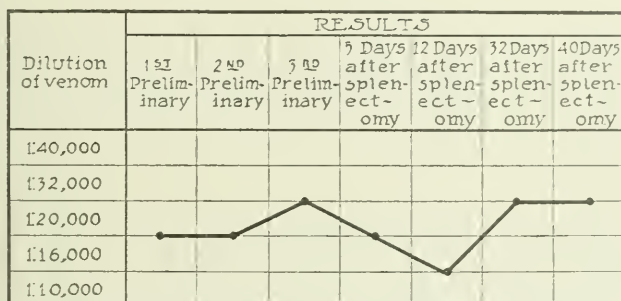
Date.	History.	Salt solution and degree of hemolysis.											
		0.3	0.325	0.35	0.375	0.4	0.425	0.45	0.475	0.5	0.525	0.55	0.575
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Feb. 1	1st preliminary.	100	100	100	100	90	80	40	30	10	5	—	—
" 8	2nd "	100	100	100	100	100	90	40	30	20	10	—	—
" 14	4 days after splenectomy.	100	100	100	90	90	80	50	40	10	5	—	—
" 23	13 " " "	100	100	100	90	60	50	30	5	—	—	—	—
" 29	19 " " "	100	100	80	30	10	10	5	5	—	—	—	—
Mar. 14	33 " " "	100	100	90	80	60	30	20	5	—	—	—	—
" 22	6 wks. " "	100	100	90	80	80	60	30	20	5	—	—	—
Apr. 20	10 " " "	100	100	100	90	80	60	60	20	10	5	—	—
May 25	4 mos. " "	100	100	100	90	90	80	40	20	10	—	—	—

The results of my experiments with the two series of dogs may be summarized as follows: (1) The resistance of the erythrocytes of normal dogs to the hemolytic action of cobra venom is likely to vary to a slight degree in different tests, as shown in the preliminary tests with Dog 4 in Series A, and Dogs 25, 26, 28, and 29 in Series B. While every effort was made to insure a uniform technique in successive experiments, it is possible that these variations were due in part at least to variations in the strength of venom or injury to the

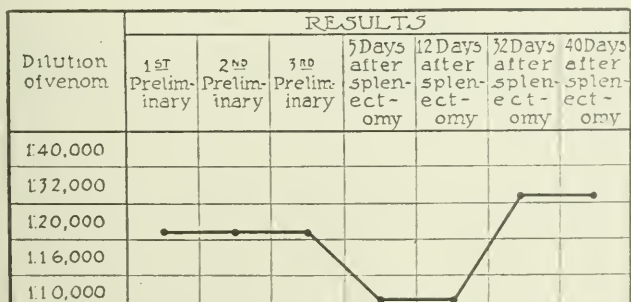




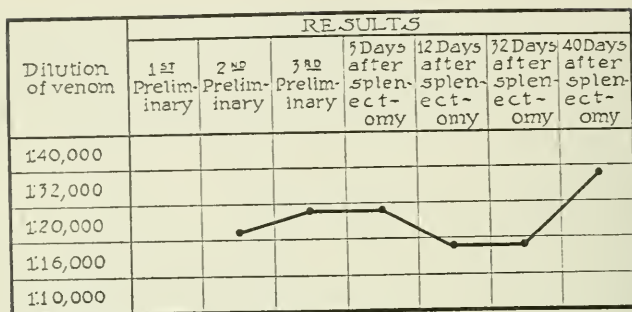
TEXT-FIG. 1. Maximal resistance of the erythrocytes of Dog 1 before and after splenectomy.



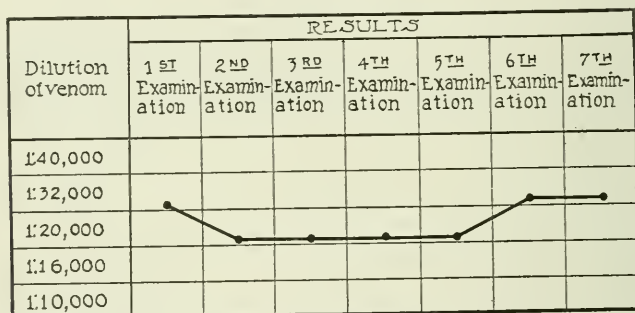
TEXT-FIG. 2. Maximal resistance of the erythrocytes of Dog 3 before and after splenectomy.



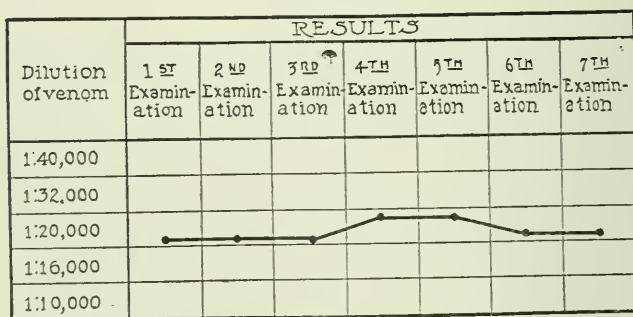
TEXT-FIG. 3. Maximal resistance of the erythrocytes of Dog 4 before and after splenectomy.



TEXT-FIG. 4. Maximal resistance of the erythrocytes of Dog 5 before and after splenectomy.

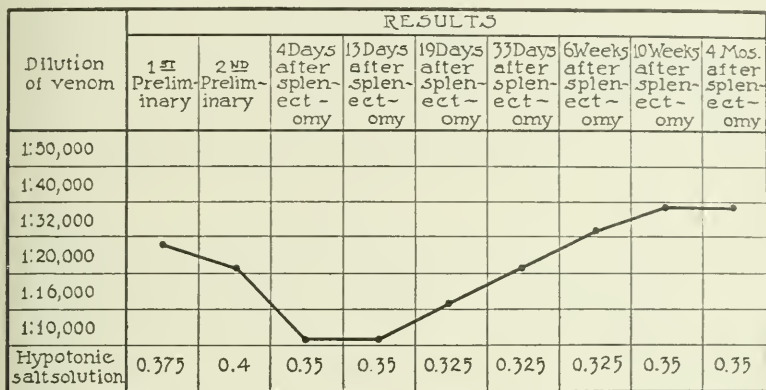


TEXT-FIG. 5. Maximal resistance of the erythrocytes of Dog 2 used as a control.

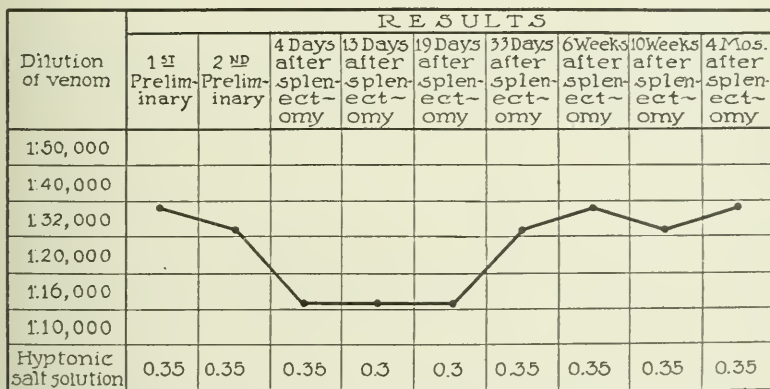


TEXT-FIG. 6. Maximal resistance of the erythrocytes of Dog 6 used as a control.

cells during the washing. At all events it would appear necessary to bear in mind these possible variations in interpreting the influence of the removal of the spleen upon the resistance of the erythrocytes.



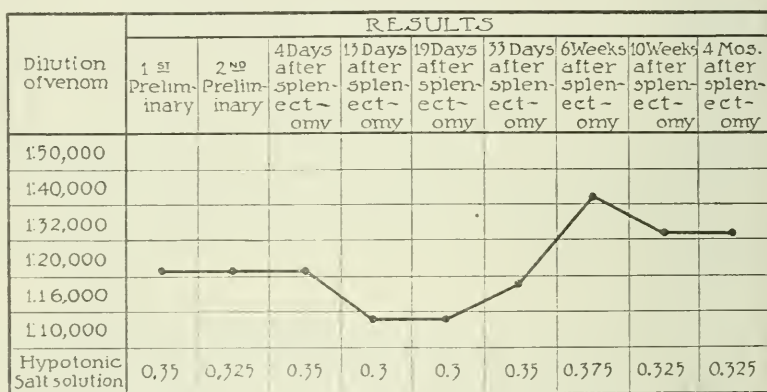
TEXT-FIG. 7. Maximal resistance of the erythrocytes of Dog 25 before and after splenectomy.



TEXT-FIG. 8. Maximal resistance of the erythrocytes of Dog 26 before and after splenectomy.

(2) On the 4th and 5th days after splenectomy the resistance of the erythrocytes to cobra venom was increased to a well marked extent in the majority of the dogs. On the 12th and 13th days after splenectomy

this increased resistance was still well marked and persisted in most instances for a period of 19 days or 3 weeks, when the erythrocytes gradually became more and more vulnerable to the influence of venom. 5 to 6 weeks after splenectomy the erythrocytes of all the dogs were as susceptible to venom as before the operation and in some instances to a greater extent. (3) Apparently the decrease of resistance to venom noted in the majority of the dogs about 4 or 5 weeks after splenectomy was coincident with an anemia, as shown by the following results of blood examinations of dogs in both series.



TEXT-FIG. 9. Maximal resistance of the erythrocytes of Dog 27 before and after splenectomy.

*Dog 3.*—32nd day after splenectomy. Hemoglobin, 16 per cent; erythrocytes, 1,072,000. Maximal resistance to venom, 1:32,000 (a decrease of resistance). (Text-fig. 2.)

*Dog 4.*—32nd day after splenectomy. Hemoglobin, 51 per cent; erythrocytes, 3,952,000. Maximal resistance to venom, 1:32,000 (a decrease of resistance). (Text-fig. 3.)

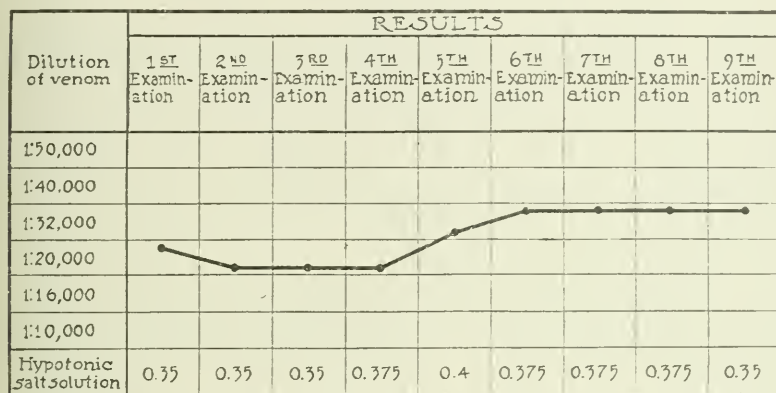
*Dog 25.*—10 weeks after splenectomy. Hemoglobin, 62 per cent; erythrocytes, 3,840,000. Maximal resistance to venom, 1:40,000 (a decrease of resistance). (Text-fig. 7.)

*Dog 26.*—10 weeks after splenectomy. Hemoglobin, 80 per cent; erythrocytes, 4,800,000. Maximal resistance to venom, 1:32,000 (normal resistance for this animal). (Text-fig. 8.)

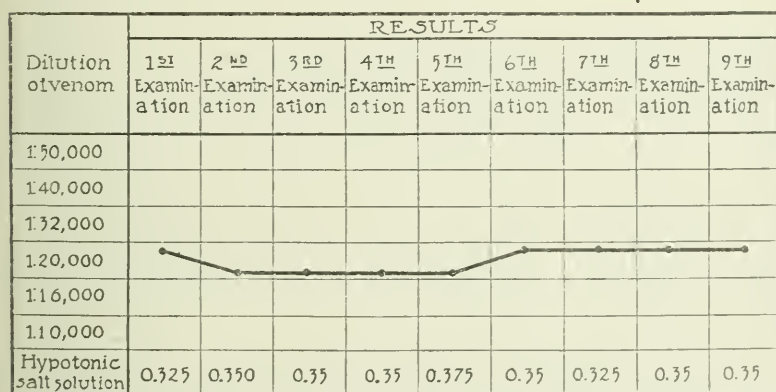
*Dog 27.*—10 weeks after splenectomy. Hemoglobin, 24 per cent; erythrocytes, 1,800,000. Maximal resistance to venom, 1:32,000 (a decrease of resistance). (Text-fig. 9.)

On the other hand, the erythrocytes of the control or non-splenectomized animals did not show a decrease of resistance to venom according to the results of blood examinations.

*Dog 6.*—32nd day. Hemoglobin, 86 per cent; erythrocytes, 5,328,000. Maximal resistance to venom, 1:20,000 (normal for this animal). (Text-fig. 6.)



TEXT-FIG. 10. Maximal resistance of the erythrocytes of Dog 28 used as a control.



TEXT-FIG. 11. Maximal resistance of the erythrocytes of Dog 29 used as a control.

*Dog 2.*—32nd day. Hemoglobin, 125 per cent; erythrocytes, 5,600,000. Maximal resistance to venom, 1:32,000 (normal for this animal). (Text-fig. 5.)

*Dog 28.*—After 10 weeks. Hemoglobin, 82 per cent; erythrocytes, 4,200,000.

Maximal resistance to venom, 1:40,000 (a decrease for this animal, probably due to distemper from which the animal was suffering). (Text-fig. 10.)

*Dog 29.*—After 10 weeks. Hemoglobin, 87 per cent; erythrocytes, 5,100,000. Maximal resistance to venom, 1:32,000 (about normal for this animal). (Text-fig. 11.)

An increased resistance to hypotonic salt solutions was shown by the three splenectomized dogs belonging to Series B (Text-figs. 7, 8, and 9). This increase of resistance on the part of the erythrocytes to hypotonic salt solutions was apparent 13 days after the operation, and in two of the dogs (Nos. 25 and 27) persisted to a slight degree throughout the 4 months' period of observation. Dog 26 showed a slightly increased resistance on the 13th and 19th days after splenectomy, and after this time the resistance returned to the point found in the tests prior to splenectomy.

The increased resistance of the erythrocytes to venom and hypotonic salt solutions developed at or about the same intervals after splenectomy, but resistance to the latter appeared to persist for a longer time.

#### CONCLUSIONS.

1. The resistance of erythrocytes of dogs to the hemolytic activity of cobra venom is increased after splenectomy.

2. This increased resistance was observed as early as 4 days after splenectomy and usually persisted for a period of about 3 weeks, when the resistance gradually decreased to normal or slightly beyond.

3. The decrease of resistance to the hemolytic activity of venom for the erythrocytes of splenectomized dogs following the primary increase is apparently coincident with the anemia following splenectomy. An intercurrent infection, such as distemper, tends to reduce the resistance of erythrocytes to venom.

4. An increased resistance of the erythrocytes to hypotonic salt solutions was found with all the splenectomized dogs in which these tests were made. Increased resistance to hypotonic salt solutions apparently persists for a longer period than the increased resistance to cobra venom.

5. As the lysis of erythrocytes by venom is dependent upon the presence of certain lipoidal substances within the cells, and as the



spleen may exercise an influence over the lipoidal contents of corpuscles and serum, it is suggested that the increased resistance of erythrocytes to the hemolytic activity of venom after splenectomy is due to alterations in the lipid content of the erythrocytes.

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# THE EFFECTS OF SERUM TREATED WITH PARARABIN.

BY EDGARD ZUNZ, M.D., AND CHARLES MOHILEVITH, M.D.

(From the Laboratories of the Physiological Institute of the University of Lausanne, Lausanne, Switzerland.)

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## INTRODUCTION.

In a previous publication<sup>1</sup> one of the writers, in collaboration with Gelat, showed that the intravenous injection of horse serum kept for 2 hours at 38°C. in the presence of one-fifth of its volume of a suspension of 0.5 per cent agar in physiological salt solution, and then separated from the agar by centrifugalization and filtration, produces in normal rabbits, when administered in adequate doses, the same symptoms that are observed after the intravenous injection of horse serum in a sero-anaphylactized rabbit.<sup>2</sup>

In these experiments it was also seen that the filtrate from the control mixture of agar and physiological salt solution sometimes produces a pronounced fall in the arterial pressure. This fall differs, however, from that caused by serum treated with agar, since in the first instance the pressure returns comparatively rapidly to its initial level, whereas in the second it remains for a long time at quite a low level.

Bordet has shown, in cooperation with one of us,<sup>3</sup> that it is possible to prepare from the relatively nitrogenous agar a product named pararabin, which constitutes a portion of the carbohydrates of agar and is practically free of nitrogen. Guinea pig serum kept for 2 or 3 hours at 37°C. in the presence of pararabin and then separated by centrifugalization and filtration from the sediment of the pararabin causes, on intravenous injection into a normal guinea pig, the same symptoms that are produced by serum similarly treated with agar; namely, tremors, dyspnea, micturition, and death after a few minutes. At autopsy, which was performed at once, dilatation of the lungs with hemorrhagic areas varying in size and number, persistence of the heart beat, and a marked retardation

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<sup>1</sup> Zunz, E., and Gelat, M., The Effects of Serum Treated with Agar, *J. Exp. Med.*, 1916, xxiv, 247.

<sup>2</sup> Arthus, M., La séro-anaphylaxie du lapin, *Arch. internat. physiol.*, 1908-09, vii, 471; 1910, ix, 156.

<sup>3</sup> Bordet, J., and Zunz, E., Production d'anaphylatoxine dans le sérum traité par de l'agar épuré de son azote (pararabine), *Z. Immunitätsforsch., Orig.*, 1915, xxiii, 42.

of the coagulation of the blood were observed. Guinea pig serum treated with pararabin also produces in normal guinea pigs the same symptoms as the intravenous injection of a heterologous serum into guinea pigs sensitized by previous subcutaneous or intraperitoneal injection of this heterologous serum.

It seemed to us important to investigate whether the identical results observed in guinea pigs after the intravenous injection of serum treated with agar or with pararabin exist also in rabbits injected with horse serum treated with agar or with pararabin.

### *Technique.*

The suspension of pararabin was prepared in exactly the same manner as the suspension of agar in the above mentioned experiments of Zunz and Gelat.<sup>1</sup> 1 gm. of pararabin was dissolved by heating in 200 cc. of physiological salt solution. This solution was distributed in test-tubes in portions of 10 cc., after which the test-tubes were sterilized and sealed. Upon cooling, a soft jelly was formed, which was transformed, by means of vigorous shaking, into a homogeneous suspension of pararabin.

To 50 cc. of horse serum were added in each experiment 10 cc. of a suspension of pararabin; *i.e.*, five parts of serum to one of pararabin. In addition, we prepared the four following control mixtures:

1. 50 cc. of normal horse serum plus 10 cc. of physiological salt solution.
2. 50 cc. of physiological salt solution plus 10 cc. of a suspension of 0.5 per cent pararabin.
3. 50 cc. of horse serum kept for 30 minutes at 56°C. plus 10 cc. of physiological salt solution.
4. 50 cc. of horse serum kept for 30 minutes at 56°C. plus 10 cc. of a suspension of 0.5 per cent pararabin.

The five mixtures were kept in well corked Erlenmeyer flasks for 2 hours at 38°C. Each mixture was then submitted to rapid centrifugalization and then to filtration. Thus, the pararabin contained in the test mixture and in the second and fourth of the control mixtures was removed. In each series of experiments, five filtrates were obtained in this way. Each filtrate was injected into the marginal vein of the ear of a normal rabbit. The respiration was registered by Verdin's recording tambours, connecting with a Marey tambour, and the carotid pressure by Verdin's registering hemodynamometer. Franck's cannula was used. As an anticoagulant a solution of 1,000 cc. of water, 65 gm. of sodium bicarbonate, and 15 gm. of magnesium sulphate was employed. In order to estimate the coagulability of the blood, we discarded the first few cubic centimeters of carotid blood, contaminated by mixture with the anticoagulant solution. The blood was collected from the carotid directly into a porcelain vessel without touching the wound. The time necessary for the formation of the first fibrin floccules and for complete coagulation was recorded.

## EXPERIMENTAL.

We performed only the two series of experiments described below. In both series intravenous injections were made in normal rabbits. ' 1

*First Series of Experiments.*

*Experiment 1.*—A rabbit weighing 2,420 gm. received two separate doses of 5 cc. of serum treated with pararabin. This dose corresponds to 2.07 cc. per kilo. (Table I.)

TABLE I.

*First Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
Before.	11.8	69	4	9.0	85
During.	12.0	70	5	9.2	84
$\frac{1}{2}$	11.7	70	6	9.25	85
1	11.4	70	7	9.4	81
2	9.6	89	8	9.5	83
3	8.8	105	9	9.8	74

*Second Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

During.	10.25	74	5	7.6	91
$\frac{1}{2}$	10.05	75	6	7.8	103
1	10.8	74	7	7.7	107
2	10.0	76	8	7.7	97
3	8.6	92	9	8.0	91
4	7.7	90	10	8.1	87

The first injection of serum treated with pararabin produced a slight temporary rise of the blood pressure, occurring during the period of injection. This was succeeded by a fall which 3 minutes after the injection attained 3.2 cm. of mercury. Thereafter the pressure gradually rose again. The respiration showed an acceleration, the maximum of which corresponded with the minimum arterial pressure.

The second serum injection produced a rise in the arterial pressure during the first minute following the injection, which then recorded 1 cm. of mercury below the initial level. It next fell, reaching its

lowest level 5 minutes after the injection. This corresponds to 3.2 cm. of mercury, if the starting point is taken as the maximum of pressure observed after the second injection; but to 4.2 cm., if the starting point is taken to be the level of pressure registered at the beginning of the experiment. After this the pressure began to rise by degrees. The respiration showed a similar acceleration after the second injection of serum treated with pararabin to that after the first. But this time the maximum respiratory rate was attained only 7 minutes after the injection and in no way corresponded to the minimum arterial pressure. No defecation occurred after the injection of serum treated with pararabin. Blood drawn from the carotid 11 minutes after the second injection showed the first fibrin formation after 35 minutes. Coagulation was complete 15 minutes later, or 50 minutes after the withdrawal of the blood.

*Experiment 2.*—A rabbit weighing 2,335 gm. received 5 cc. of serum treated with pararabin, followed 11 minutes later by 10 cc. of the same serum. These doses correspond respectively to 2.14 and 4.28 cc. per kilo. (Table II.)

TABLE II.

*First Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
Before.	9.8	115	4	9.5	115
During.	10.0	113	5	9.0	115
$\frac{1}{2}$	9.95	115	6	8.6	104
1	9.8	119	7	8.6	83
2	9.2	109	8	8.45	72
3	9.25	120	9	8.45	72
			10	8.45	72

*Second Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

During.	9.2	67	5	8.5	56
$\frac{1}{2}$	9.0	65	6	8.4	56
1	9.0	59	7	8.4	55
2	9.0	59	8	8.1	55
3	8.9	53	9	8.0	56
4	8.65	52	10	8.0	56



During the injection of serum treated with pararabin the arterial pressure rose slightly. It then gradually fell until 8 minutes after the injection it had reached a level 1.35 cm. of mercury below the initial level. The pressure was maintained at this level for 2 minutes. A second injection equal to double the first dose of the same serum treated with pararabin caused the pressure to rise 0.75 cm. of mercury, after which it again declined. 9 minutes after the second injection it had reached a level 1.8 cm. of mercury below the original level. The respiratory rate was comparatively rapid at the beginning of the experiment, but it began to diminish 6 minutes after the first injection. This retardation stopped at about the 8th minute following the injection. It was resumed directly after the second injection and stopped 4 minutes after the new injection. After the second injection there was an expulsion of twelve scybala. Blood drawn from the carotid 11 minutes after the second injection of serum treated with pararabin showed the first fibrin formation after 27 minutes; coagulation was complete 3 minutes later, or 30 minutes after the withdrawal of the blood.

*Experiment 3.*—A rabbit weighing 1,820 gm. received 10 cc. of serum treated with pararabin, corresponding to 5.49 cc. per kilo. (Table III.)

TABLE III.

*Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
Before.	10.6	77	5	9.6	99
During.	10.9	67	6	9.5	93
$\frac{1}{2}$	10.7	69	7	9.6	82
1	10.3	77	8	9.6	64
2	10.1	92	9	9.7	67
3	9.6	96	10	9.7	69
4	9.6	88	11	9.8	65
			12	9.8	64

The intravenous injection of serum treated with pararabin produced in this case, after a slight temporary rise of blood pressure, a gradual fall which 6 minutes after the beginning of the injection reached a level only 1.1 cm. of mercury below the initial level. After

this the pressure rose again slowly. 2 minutes after the injection the respiratory rate began to increase, and this lasted for 5 minutes. The respiratory rate showed two maxima, the first occurring 3 minutes after the beginning of the injection, and the second 2 minutes later. There was no defecation. Unfortunately the time required for the coagulation of the blood was not recorded.

*Experiment 4.*—A rabbit weighing 1,915 gm. received 5 cc. of a filtrate of pararabin and physiological salt solution; 15 minutes later 5 cc. of a filtrate of serum and physiological salt solution were given. This corresponds to 2.61 cc. per kilo. (Table IV.)

TABLE IV.

*Injection of Filtrate of Pararabin and Physiological Salt Solution (Control).*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	cm. Hg.	per min.	min.	cm. Hg.	per min.
Before.	9.0	44	6	8.6	44
During.	9.0	43	7	8.45	45
$\frac{1}{2}$	9.0	45	8	8.5	46
1	8.95	46	9	8.3	46
2	8.9	45	10	8.3	46
3	8.9	44	11	8.15	45
4	8.8	48	12	8.0	45
5	8.7	44	13	8.1	45
			14	8.0	46

*Injection of Filtrate of Normal Horse Serum and Physiological Salt Solution (Control).*

During.	8.55	46	3	8.15	43
$\frac{1}{2}$	8.5	44	4	8.1	41
1	8.45	44	5	8.05	42
2	8.15	43	6	7.95	43

The arterial pressure gradually fell after the injection of the filtrate of pararabin and physiological salt solution. This fall attained its maximum (1 cm. of mercury) 12 minutes after the injection. After the intravenous injection of a filtrate of serum and physiological salt solution, the pressure rose 0.55 cm. of mercury, then gradually fell again. 6 minutes after the second injection the pressure had reached 1.05 cm. of mercury below the initial level. At this point the experiment was unfortunately interrupted.

The respiratory rate remained unmodified during the experiment. There was an expulsion of seventeen scybala after the injection of the filtrate of pararabin and physiological salt solution. Blood drawn from the carotid 7 minutes after the second injection showed the first fibrin formation at the end of 12 minutes; coagulation was complete 6 minutes later, or 18 minutes after the withdrawal of the blood.

*Experiment 5.*—A rabbit weighing 1,830 gm. received 5 cc. of the filtrate of pararabin and serum heated to 56°C., and 7 minutes later 5 cc. of filtrate of physiological salt solution and serum heated to 56°C. This corresponds to 2.73 cc. per kilo. (Table V.)

TABLE V.

*Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Pararabin (Control).*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	cm. Hg.	per min.	min.	cm. Hg.	per min.
Before.	10.4	42	3	10.2	43
During.	10.5	46	4	10.2	44
$\frac{1}{2}$	10.4	44	5	10.2	52
1	10.3	43	6	10.2	49
2	10.2	44			

*Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Physiological Salt Solution (Control).*

During.	10.3	49	6	9.7	37
$\frac{1}{2}$	10.1	39	7	9.4	36
1	10.1	38	8	9.4	38
2	10.0	40	9	9.4	35
3	10.0	43	10	9.4	35
4	9.9	39	11	9.3	35
5	9.8	39	12	9.3	35

The injection of the filtrate of pararabin and serum heated to 56°C. produced no appreciable modification of the arterial pressure or of the respiratory rate. There was no defecation.

The injection of the filtrate of physiological salt solution and serum heated to 56°C. caused a depression of the arterial pressure, which 11 minutes later reached 1 cm. of mercury. The respiratory rate was slightly retarded. There was an expulsion of five scybala.

Blood drawn from the carotid 13 minutes after the second injec-

tion showed the first fibrin formation at the end of 15 minutes. Coagulation was complete 3 minutes later, or 18 minutes after the withdrawal of blood.

Table VI summarizes the results obtained in the first series of experiments.

TABLE VI.

*Summary of First Series of Experiments.*

Experiment No.	Liquid injected. Filtrate from mixture of.	Amount per kg. of liquid injected.	Effects of injection.				
			Blood pressure.	Respiratory rate.	Scybal.	Time of coagulation after blood withdrawal.	
						First fibrin formation.	Complete coagulation.
		cc.				min.	min.
1	Serum plus par- arabin.	2.07	Temporary rise; then fall of 3.2 cm. Hg.	Acceleration.	0	—	—
	“ “ “	2.07	Temporary rise; then fall of 3.2 cm. Hg.	“	0	35	50
2	“ “ “	2.14	Temporary rise; then fall of 1.35 cm. Hg.	Retardation.	0	—	—
	“ “ “	4.28	Temporary rise; then fall of 1.8 cm. Hg.	“	12	27	30
3	“ “ “	5.49	Temporary rise; then fall of 1.1 cm. Hg.	Acceleration.	0	—	—
4	Physiological salt solution plus par- arabin.	2.61	Immediate fall of 1.0 cm. Hg.	No change.	0	—	—
	Physiological salt solution plus ser- um.	2.61	Temporary rise; then fall of 1.05 cm. Hg.	“ “	17	12	18
5	Serum heated to 56°C. plus par- arabin.	2.73	No change.	“ “	0	—	—
	Serum heated to 56°C. plus physio- logical salt solu- tion.	2.73	Immediate fall of 1.0 cm. Hg.	Retardation.	5	15	18

From results obtained with control filtrates, it appears that the fall of pressure must exceed 1 cm. of mercury in order that it may be considered as a manifestation of anaphylactic shock. No importance should be attributed to the expulsion of scybala. But, on the other hand, every delay in the blood coagulation and every acceleration of the respiratory rate must be considered as a result of anaphylactic shock.

Thus, if we admit the above assumptions, it is seen that the injection of a filtrate of serum kept at 38°C. in the presence of pararabin produces a distinct anaphylactic shock when administered in doses of 2.07 cc. per kilo (Experiment 1), and that this effect occurs after two successive injections. No definite statement can be made about larger doses.

### *Second Series of Experiments.*

The second series, like the first, represents results obtained with five normal rabbits.

*Experiment 6.*—A rabbit weighing 2,335 gm. was injected with 1 cc. of serum treated with pararabin, and 11 minutes later with 2 cc. of the same serum. These doses correspond respectively to 0.43 and 0.86 cc. per kilo. (Table VII.)

TABLE VII.

#### *First Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
Before.	11.8	53	4	10.8	46
During.	11.6	52	5	10.7	46
$\frac{1}{2}$	12.0	50	6	11.0	44
1	12.0	45	7	11.0	49
2	11.0	48	8	10.8	48
3	10.7	47	9	10.7	48
			10	11.2	48

#### *Second Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
During.	11.0	48	3	10.7	46
$\frac{1}{2}$	10.8	48	4	10.6	45
1	11.0	48	5	10.5	45
2	11.0	46	6	10.4	45

The injection of 0.43 cc. per kilo of the serum treated with pararabin produced a fall of the arterial pressure which after 3 minutes had reached 1.1 cm. of mercury. This fall was preceded by a slight rise of pressure, which followed upon a first, very slight reduction, occurring simultaneously with the injection. The pressure then gradually rose, although a slight fall occurred during the 5th and 9th minutes. The second injection of serum treated with pararabin also showed a fall of the arterial pressure. After the experiment was discontinued, that is, 6 minutes after the injection, this fall had reached a level of 0.8 cm. of mercury.

The respiratory rate tended to diminish slightly after each of the two injections, but this phenomenon was hardly perceptible. Defecation occurred after the second injection.

Blood taken from the carotid 7 minutes after the second injection showed the first fibrin formation at the end of 18 minutes; coagulation was complete after 7 minutes more; that is, 25 minutes after the withdrawal of the blood.

*Experiment 7.*—A rabbit weighing 2,230 gm. received 2 cc. of serum treated with pararabin, or 0.89 cc. per kilo. (Table VIII.)

TABLE VIII.

*Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
Before.	11.7	70	9	9.0	57
During.	11.0	69	10	9.0	60
$\frac{1}{2}$	10.7	67	11	9.1	59
1	10.3	73	12	9.15	58
2	10.0	67	13	9.25	56
3	9.7	79	14	9.4	54
4	9.4	82	15	9.3	54
5	9.2	76	16	9.3	54
6	9.2	72	17	9.4	50
7	9.2	69	18	9.3	52
8	9.0	67	19	9.3	52

The arterial pressure fell 2.7 cm. of mercury in 8 minutes. 11 minutes after the injection it rose again slowly.



A slight acceleration of the respiratory rate was noticeable 3 minutes after the injection, reaching its maximum at the end of the 4th minute. After the 9th minute the respiratory rate showed signs of diminishing. Defecation occurred after this injection.

Blood taken from the carotid 20 minutes after the injection showed the first fibrin formation after 25 minutes. Coagulation was not complete until 25 minutes later; *i.e.*, 50 minutes after the withdrawal of the blood.

*Experiment 8.*—A rabbit weighing 2,020 gm. received 5 cc. of serum treated with pararabin, or 2.47 cc. per kilo. (Table IX.)

TABLE IX.

*Injection of Filtrate of Normal Horse Serum Treated with Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
Before.	9.4	57	9	9.4	53
During.	9.0	47	10	9.5	55
$\frac{1}{2}$	9.4	52	11	9.3	53
1	9.0	52	12	9.3	51
2	9.6	52	13	9.2	52
3	9.8	50	14	9.3	52
4	9.6	52	15	9.3	53
5	9.4	58	16	9.3	54
6	9.6	56	17	9.3	53
7	9.7	60	18	9.2	54
8	9.5	60	19	9.3	54
			20	9.4	54

The injection of 2.47 cc. per kilo of serum treated with pararabin failed to show any definite effects on the pressure. The pressure first of all fell 0.4 cm. of mercury, then rose 0.4 cm.; it then fell 0.4 cm. again, after which it rose 0.8 cm. of mercury. Thereafter it fluctuated between 0.2 cm. of mercury above and below the initial level.

The respiratory rate first slightly diminished, then 7 to 8 minutes after the injection rose a little above the initial figure, after which it fell slightly. No defecation took place.

Blood taken from the carotid 21 minutes after the injection showed the first fibrin formation after 30 minutes. Coagulation was not

complete until 20 minutes later; that is, 50 minutes after the withdrawal of the blood.

*Experiment 9.*—A rabbit weighing 1,970 gm. was injected with 2 cc. of the filtrate of pararabin and physiological salt solution; 11 minutes later it received 2 cc. of the filtrate of pararabin and serum. This corresponds to 1.02 cc. per kilo for each injection. (Table X.)

TABLE X.

*Injection of Filtrate of Pararabin and Physiological Salt Solution (Control).*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>	<i>min.</i>	<i>cm. Hg.</i>	<i>per min.</i>
Before.	10.5	42	4	10.0	42
During.	10.6	43	5	9.9	35
$\frac{1}{2}$	10.6	44	6	10.0	39
1	10.2	42	7	10.0	38
2	10.3	42	8	10.2	40
3	10.1	39	9	10.2	45
			10	10.2	48

*Injection of Filtrate of Normal Horse Serum and Pararabin.*

During.	10.3	37	4	10.3	44
$\frac{1}{2}$	10.0	40	5	10.5	45
1	9.8	42	6	10.4	48
2	9.9	44	7	10.4	46
3	10.2	46	8	10.2	45
			9	10.0	45

The injection of the filtrate of pararabin and physiological salt solution at first produced a slight temporary rise of the arterial pressure, which was followed after 5 minutes by a fall of 0.7 cm. of mercury. After this the pressure slowly rose again. The injection of the filtrate of pararabin and serum also produced a slight and transient rise of the pressure, which occurred during the injection. Then for the space of 1 minute the pressure fell 0.5 cm. of mercury, and rose to the initial level 4 minutes later, after which it gradually fell again. At the expiration of the experiment, which occurred 9 minutes after the second injection, a new fall of the arterial pressure had occurred, corresponding to 0.5 cm. of mercury; this fall would perhaps subsequently have continued.

The respiratory rate decreased slightly after the injection of the filtrate of pararabin and physiological salt solution. Later it returned to the initial figure and even slightly exceeded it. During the injection of the filtrate of pararabin and serum the respiratory rate diminished; later it regained and for a time even slightly exceeded the initial figure. These modifications were not very great. There was an expulsion of forty-five scybala after the second injection.

Blood taken from the carotid 10 minutes after the second injection showed the first fibrin formation at the end of 45 minutes. Coagulation was complete 10 minutes later; that is, 55 minutes after the withdrawal of the blood.

*Experiment 10.*—A rabbit weighing 2,140 gm. received successively injections of each of the following (Table XI):

1. 2 cc. of a filtrate of serum and pararabin.
2. 2 " " " " " " " heated to 56°C. and pararabin.
3. 2 " " " " " " " " 56° " " physiological salt solution.

TABLE XI.

*Injection of Filtrate of Normal Horse Serum and Pararabin.*

Time after injection.	Arterial pressure.	Respiratory rate.	Time after injection.	Arterial pressure.	Respiratory rate.
min.	cm. Hg.	per min.	min.	cm. Hg.	per min.
Before.	10.7	40	4	10.2	35
During.	10.9	38	5	9.4	39
$\frac{1}{2}$	10.6	35	6	9.2	36
1	10.6	35	7	9.0	43
2	10.4	38	8	8.4	42
3	10.0	38	9	8.8	43

*Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Pararabin (Control).*

During.	9.2	42	3	9.6	42
$\frac{1}{2}$	9.4	42	4	9.2	43
1	9.6	40	5	9.4	45
2	9.4	39	6	9.2	44
			7	9.3	49

*Injection of Filtrate of Normal Horse Serum Heated to 56°C. and Physiological Salt Solution (Control).*

During.	9.5	45	3	9.8	48
$\frac{1}{2}$	9.4	45	4	10.0	49
1	9.8	47	5	9.9	50
2	9.8	48	6	9.9	48

The injection of the filtrate of serum and pararabin produced first, simultaneously with the injection, a temporary and insignificant rise of the arterial pressure, and then a fall which reached 2.3 cm. of mercury 8 minutes after the injection. But, on the other hand, the injection of the filtrates of serum heated to 56°C. and mixed either with pararabin or with physiological salt solution produced rises in the arterial pressure attaining, respectively, 0.4 cm. of mercury after 1 minute and 0.5 cm. after 4 minutes.

The respiratory rate did not suffer any appreciable modifications, although it tended to diminish slightly after the injection of the filtrate of serum and pararabin; it also showed signs of increasing after the injection of the filtrates of serum heated to 56°C. and mixed either with pararabin or physiological salt solution.

There was an expulsion of five scybala following the injection of the filtrate of pararabin and serum heated to 56°C., and an expulsion of seven scybala after the injection of the filtrate of physiological salt solution and heated serum.

Blood drawn from the carotid 7 minutes after the third injection showed the first fibrin formation after 12 minutes. Coagulation was complete 5 minutes later, or 17 minutes after the withdrawal of the blood.

Table XII shows the results of the second series of experiments.

In the second series of experiments we unfortunately omitted to note the action of the filtrate derived from the serum and physiological salt solution. But the results obtained with the three other control liquids lead us to assume that in all probability this mixture would not have caused a fall in the arterial pressure greater than 0.5 cm. of mercury after a dose of approximately 1 cc. per kilo. Such, indeed, was observed in Experiment 9, in the case of the filtrate of physiological salt solution and pararabin. The filtrates prepared with the serum heated to 56°C. caused a corresponding rise in the arterial pressure.

At all events, in Experiments 7 and 10, the serum treated with pararabin produced a distinct fall in the arterial pressure after a dose of approximately 1 cc. per kilo. Experiments 6 and 9 did not show this characteristic fall as a result of the same dose. With a dose of 2.47 cc., *i.e.*, one slightly greater than that which, in the first series

TABLE XII.

*Summary of Second Series of Experiments.*

Experiment No.	Liquid injected. Filtrate from mixture of.	Amount per kg. of liquid in- jected.	Effects of injection.				
			Blood pressure.	Respiratory rate.	Scybalæ.	Time of coagulation after blood withdrawal.	
						First fibrin formation.	Complete co- agulation.
		cc.				min.	min.
6	Serum plus par- arabin.	0.43	Temporary rise; then fall of 1.1 cm. Hg.	Very slight re- tardation.	0	—	—
	“ “ “	0.86	Temporary rise; then fall of 0.8 cm. Hg.	“ “ “	9	18	25
7	“ “ “	0.89	Fall of 2.7 cm. Hg.	Slight acceler- ation.	5	25	50
8	“ “ “	2.47	Immediate modi- fications in both directions.	Insignificant changes in both direc- tions.	0	30	50
9	Physiological salt solution plus par- arabin.	1.02	Temporary rise; then fall of 0.7 cm. Hg.	Slight retardation.	11	—	—
	Serum plus par- arabin.	1.02	Temporary rise; then fall of 0.5 cm. Hg.	“ “	45	45	55
10	“ “ “	0.93	Temporary rise; then fall of 2.3 cm. Hg.	No change.	0	—	—
	Heated serum plus pararabin.	0.93	Rise of 0.4 cm. Hg.	“ “	5	—	—
	Heated serum plus physiological salt solution.	0.93	Rise of 0.5 cm. Hg.	“ “	7	12	17

of experiments, brought about a fall of pressure of 3.2 cm. of mercury and more (Experiment 1), the intravenous injection of serum treated with pararabin was seen to exert no effects on the arterial pressure.

We noted a retardation of the coagulation after each injection



of serum treated with pararabin. This phenomenon was most marked in Experiment 9, after the injection of a dose of 1.02 cc.

Intravenous injections of control filtrates (Experiment 10) sometimes caused the expulsion of several scybala. It should be stated that the only manifestation of anaphylactic shock in this connection is the expulsion of numerous scybala. This occurred in only one case out of five (Experiment 9).

The respiratory rate showed an increase, to a comparatively slight extent, in one out of five experiments (Experiment 7), which represented a dose of 0.89 cc. of serum treated with pararabin.

#### DISCUSSION.

If one compares the results obtained in the two series of experiments, it is seen that the quantity of filtrate derived from the serum which had remained at 38°C. in the presence of pararabin necessary to produce anaphylactic shock, varies according to the proportion of serum and of pararabin used, even when apparently identical experimental conditions are observed. This quantity is less in the second series than in the first. Each series appears to have an optimum dose. When the serum treated with pararabin is injected intravenously in quantities either greater or less than the optimum amount, it exerts a slighter action than the optimum dose, and if it varies considerably from this optimum, it may even fail to produce any anaphylactic shock whatever in normal rabbits.

On the other hand, if in a similar series of experiments the same dose of serum is treated with pararabin, the phenomena of anaphylactic shock are sometimes either completely or nearly completely absent (as in Experiment 6), whereas at other times they can be distinctly observed (Experiment 7). This is not surprising, in view of the fact that it has long been known that in all investigations regarding anaphylaxis there exist great differences between individual animals subjected to the same treatment.

According to our two series of experiments, the fall in the pressure effected by the injection of the filtrate of physiological salt solution and pararabin has always been inconsiderable (0.7 cm. of mercury in Experiment 9, 1 cm. in Experiment 4), whereas the contrary has some-



times been the case after the intravenous injection into a normal rabbit of a filtrate derived from a mixture of agar and physiological salt solution. In the third series of experiments described by Zunz and Gelat, this filtrate when injected in a dose of 2.22 cc. per kilo caused in 4 minutes a fall in the arterial pressure of 7.1 cm. of mercury, and represented a much greater shock even than that observed in the series of experiments with serum treated with agar. But the rate of pressure immediately increased rapidly, contrary to the phenomenon observed after the intravenous injection of serum which had remained in the presence of agar at 38°C. for 2 hours. In the three other cases observed by Zunz and Gelat, the fall of pressure was less accentuated after the intravenous injection of the filtrate of agar and physiological salt solution; *i.e.*, 1.3 cm. of mercury in 7 minutes in Experiment 4, 2.9 cm. of mercury in 8 minutes in Experiment 8, and 1.3 cm. of mercury in 6 minutes in Experiment 12.

In all of the experiments none of the other phenomena char-

## ERRATUM.

227<sup>2</sup>

On page 227, Vol. XXV, No. 2, February 1, 1917, line 20, for 2 or 3 minutes and 20 or 30 minutes.

On page 228, lines 28, 29, and 30 should read: *It should also be mentioned, has been shown by the experiments of Bordet and Zunz with guinea pigs, that it better to employ homologous serum; that is, rabbit serum.*

the retardation of carotid coagulation constitute the essential characteristics of shock. But, on the other hand, it is not always possible to detect acceleration of the respiratory rate and excitability of intestinal activity.

As in the experiments on the guinea pig made by Bordet in conjunction with one of the writers, serum treated with pararabin appears to produce a less intense anaphylactic shock in the rabbit than serum treated with agar. But it is not certain that this is really the case. For there is no evidence that the proportion of one volume of a suspension of 0.5 per cent of pararabin to five volumes of fresh horse serum is the optimum. It is possible that a more intense anaphylactic shock

of serum treated with pararabin. This phenomenon was most marked in Experiment 9, after the injection of a dose of 1.02 cc.

Intravenous injections of control filtrates (Experiment 10) sometimes caused the expulsion of several scybala. It should be stated that the only manifestation of anaphylactic shock in this connection is the expulsion of numerous scybala. This occurred in only one case out of five (Experiment 9).

The respiratory rate showed an increase, to a comparatively slight extent, in one out of five experiments (Experiment 7), which represented a dose of 0.89 cc. of serum treated with pararabin.

#### DISCUSSION.

If one compares the results obtained in the two series of experiments, it is seen that the quantity of filtrate derived from the serum which had remained at 38°C. in the presence of pararabin necessary

lactic shock are sometimes either completely or nearly completely absent (as in Experiment 6), whereas at other times they can be distinctly observed (Experiment 7). This is not surprising, in view of the fact that it has long been known that in all investigations regarding anaphylaxis there exist great differences between individual animals subjected to the same treatment.

According to our two series of experiments, the fall in the pressure effected by the injection of the filtrate of physiological salt solution and pararabin has always been inconsiderable (0.7 cm. of mercury in Experiment 9, 1 cm. in Experiment 4), whereas the contrary has some-

times been the case after the intravenous injection into a normal rabbit of a filtrate derived from a mixture of agar and physiological salt solution. In the third series of experiments described by Zunz and Gelat, this filtrate when injected in a dose of 2.22 cc. per kilo caused in 4 minutes a fall in the arterial pressure of 7.1 cm. of mercury, and represented a much greater shock even than that observed in the series of experiments with serum treated with agar. But the rate of pressure immediately increased rapidly, contrary to the phenomenon observed after the intravenous injection of serum which had remained in the presence of agar at 38°C. for 2 hours. In the three other cases observed by Zunz and Gelat, the fall of pressure was less accentuated after the intravenous injection of the filtrate of agar and physiological salt solution; *i.e.*, 1.3 cm. of mercury in 7 minutes in Experiment 4, 2.9 cm. of mercury in 8 minutes in Experiment 8, and 1.3 cm. of mercury in 6 minutes in Experiment 12.

In both series of experiments none of the other phenomena characteristic of anaphylactic shock were produced after the introduction into the blood stream of the filtrate of pararabin and physiological salt solution.

It sufficed to heat the serum to 56°C. for 2 or 30 minutes in order that the treatment with pararabin should fail to give it the power of producing the symptoms of anaphylactic shock when injected intravenously into a normal rabbit.

To sum up, serum treated with pararabin causes, as does serum treated with agar, the phenomena of anaphylactic shock when injected into normal rabbits. In both cases the fall in arterial pressure and the retardation of carotid coagulation constitute the essential characteristics of shock. But, on the other hand, it is not always possible to detect acceleration of the respiratory rate and excitability of intestinal activity.

As in the experiments on the guinea pig made by Bordet in conjunction with one of the writers, serum treated with pararabin appears to produce a less intense anaphylactic shock in the rabbit than serum treated with agar. But it is not certain that this is really the case. For there is no evidence that the proportion of one volume of a suspension of 0.5 per cent of pararabin to five volumes of fresh horse serum is the optimum. It is possible that a more intense anaphylactic shock

would be produced by diminishing or augmenting the proportion of pararabin.

At all events, as the filtrate derived from the control mixture of pararabin and physiological salt solution causes a much slighter fall in the arterial pressure than the filtrate of physiological salt solution and agar, it follows that pararabin and not agar must be employed in subsequent investigations.

The best proportions of serum and pararabin, respectively, and the optimum duration of contact at  $38^{\circ}\text{C}$ . should be determined first. This would necessitate numerous series of experiments. In each one it would be necessary to inject into different rabbits 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, and 2 cc. of serum treated with pararabin, as well as greater or less quantities, according to the results obtained. In order to start from well established facts and to avoid the individual factor which is so considerable in all investigations of anaphylaxis, it would be well to inject each dose into at least three rabbits. This would necessitate a large number of animals, which, unfortunately, we are unable to obtain under present conditions.

When the appropriate dose per kilo of serum treated with pararabin has been determined, the next step would be to make the necessary investigations of similar quantities of the control filtrates, at least three normal rabbits being employed for each liquid. It is also necessary that only one liquid (serum treated with pararabin or one of the control filtrates) should be injected into each animal. Only by this means can reliable results be obtained, for the experiments described above have demonstrated the necessity of these precautions.

It should also be mentioned, as has been shown by the experiments of Bordet and Zunz<sup>with guinea pigs</sup>, that it is better to employ the homologous serum; ~~in the experiments with guinea pigs.~~ *that is, rabbit serum.*

Although our experiments cannot be looked upon as other than purely preliminary attempts, nevertheless they confirm the results obtained by Bordet and Zunz in guinea pigs injected with serum kept at  $38^{\circ}\text{C}$ . in the presence of pararabin and subsequently separated from it.

## SUMMARY.

Horse serum, kept for 2 hours at 38°C. in the presence of one-fifth its volume of a suspension of 0.5 per cent of pararabin in physiological salt solution and then freed by means of centrifugalization and filtration from the pararabin produced when injected in appropriate doses into a normal rabbit, a considerable and prolonged fall of the blood pressure, a distinct retardation in the coagulation of the carotid blood, and sometimes, in addition, acceleration of the respiratory rate and the expulsion of numerous scybala; that is, the various symptoms observed to occur after the intravenous injection of horse serum in sero-anaphylactized rabbits.

If the horse serum is first heated for 30 minutes to 56°C. and then treated with pararabin in the manner described above, the intravenous injection of this serum into a normal rabbit exerts no more action on the arterial pressure, the coagulability of the arterial blood, the respiratory rate, and the intestinal activity, than does normal horse serum when introduced into the vein of a normal rabbit.





## STUDIES ON THE BLOOD PROTEINS.

### II. THE ALBUMIN-GLOBULIN RATIO IN EXPERIMENTAL INTOXICATIONS AND INFECTIONS.

BY S. H. HURWITZ, M.D., AND G. H. WHIPPLE, M.D.

*(From the George Williams Hooper Foundation for Medical Research of the University of California Medical School, San Francisco.)*

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The changes which may occur in the partition of the blood protein fractions in different pathological conditions have been given little systematic study. For the most part, the recorded observations deal with the alteration in the albumin-globulin ratio in infections and in various immune states (1).

The present study was undertaken to determine whether the rise in the serum globulins reported in immunized animals occurred also during the immunity or tolerance to proteose intoxication, which develops in dogs with long-standing intestinal loops or after the injection of repeated, small doses of pure proteose. We have studied also the blood changes in animals which were being observed in connection with other experiments. In some of these animals symptoms of intoxication developed after the onset of peritonitis or the production of a pancreatitis or pleurisy,—conditions which have been shown to possess certain features in common with intestinal obstruction (2).

Our experimental observations have shown that the intoxication which develops after an acute or chronic obstruction of the intestine is in most instances associated with definite changes in the blood proteins. In a simple obstruction with toxic death and a rise in the non-coagulable nitrogen of the blood the serum globulins may rise to double their initial value within 48 hours after the production of the obstruction. And in the majority of such instances the globulins continue high until death. Similarly, animals with a chronic obstruction show a rise in the serum globulins. In these animals the globu-

lin increase takes place more slowly, but it may be of greater magnitude, and may show a tendency to return to its initial value.

Some of the most important observations have come from a study of the blood of animals with some of the complications met with in these loop experiments,—peritonitis, rupture of the loop, and overwhelming intoxication. In these experiments, the globulin content of the serum usually rises with marked rapidity and to the highest point. The blood of some of the animals may show a complete inversion of the normal albumin-globulin ratio. We have found this immediate rise in the blood globulins to be so constantly present in these complications that we believe its occurrence to be of value in the differential diagnosis of acute conditions associated with the sudden liberation of a toxic exudate. This observation is in harmony with previously recorded experiments (1), which have demonstrated that a number of inflammatory conditions with tissue destruction and pus formation are accompanied by a rise in the blood globulins. And from the present experiments we have additional evidence that an increase in the globulins may occur even when the inflammatory conditions are not the result of bacterial invasion, although it would appear that a combination of both factors,—bacteria and inflammatory irritants gives rise to greater alterations in the ratio.

Injections of small immunizing doses of toxic proteose, on the other hand, do not produce any marked alteration either in the percentage of the serum proteins or in the normal partition of the albumin and globulin fractions. The changes observed in the loop experiments may, however, result if the dosage is large and the attending intoxication profound.

From these experiments we have, furthermore, additional evidence that no parallelism exists between a rise in the blood globulins and the development of an immunity or tolerance. That such a tolerance resulted from the repeated proteose injections is clear from the fact that the animals were resistant to the injection of larger doses of this toxic substance; and yet observations upon the blood failed to disclose an increase in the globulin content. An occasional increase in the percentage of globulin did occur, but this was usually transitory and small.

*Methods.*

Dogs were used in all the experiments. The operations were done under surgical anesthesia and with the usual aseptic technique. A description of the experimental conditions studied has been given in previous communications (3).

Specimens of blood were obtained from the jugular vein previous to a feeding in order to get a clear serum, and analyses were made prior to or on the day of operation and at regular intervals thereafter.

All the determinations of the albumin, globulin, and non-protein fractions recorded in the columns of the tables were made by the micro-refractometric method of Robertson (4). The advantages of this method for these studies over those used heretofore have been discussed in a previous paper (1).

## EXPERIMENTAL.

*The Serum Proteins of Normal Dogs.*

The percentage of total protein and the relative proportions of albumin and globulin present in normal dog serum have not been extensively studied by the more recent and more accurate methods. The high globulin figures obtained by Lewinski<sup>1</sup> (5) are not in accord with the more recent percentages obtained by the micro-refractometric method. The percentages obtained by Robertson and his coworkers (6) as well as our own are considerably lower (Table I). We have found the average total protein percentage obtained from a study of thirty-five normal dogs to be 6.1 per cent, of which the globulin fraction constituted 1.5 per cent. These figures are in accord with those recently obtained by Jewett (6) in a study of a smaller number of sera by the same method.

<sup>1</sup> According to Lewinski, the percentage composition of serum proteins in normal dogs is as follows: total protein, 6.03 per cent; serum albumin, 3.17 per cent; serum globulin, 2.26 per cent. From his figures it is clear that the serum globulins would constitute 42 per cent of the total proteins, which is 17 per cent higher than the percentage obtained by us.

TABLE I.

*Albumin, Globulin, and Total Protein in the Blood Serum of Normal Dogs.*

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.*
		per cent	per cent	per cent	per cent	per cent	per cent	
1	Nov. 26	6.2	4.8	1.4	76	24	1.5	3.1
2	" 3	5.6	4.1	1.5	71	26	2.3	2.7
3	" 3	5.7	3.6	2.1	63	37	2.0	1.7
4	" 6	6.3	5.1	1.2	80	20	1.7	4.0
5	" 10	5.8	4.4	1.4	76	24	2.1	3.1
6	" 22	5.9	4.2	1.7	70	30	1.7	2.3
7	" 29	6.3	5.2	1.1	83	17	1.7	4.8
8	" 29	6.2	5.0	1.2	80	20	1.7	4.0
9	" 29	6.4	5.3	1.1	82	18	1.6	4.5
10	Dec. 3	6.0	4.1	1.9	70	30	1.2	2.3
11	" 9	6.5	5.2	1.3	82	18	1.6	4.5
12	" 20	6.6	5.4	1.2	81	19	1.5	4.2
13	Jan. 25	5.9	4.0	1.9	68	32	1.8	2.1
14	" 28	6.6	5.6	1.0	85	15	2.2	5.6
15	Feb. 3	4.9	3.8	1.1	77	23	1.8	3.3
16	Mar. 13	4.7	4.0	0.7	85	15	2.4	5.6
17	Jan. 26	6.3	4.7	1.6	74	26	1.9	2.8
18	Feb. 2	5.7	3.7	2.0	65	35	1.7	1.9
19	" 9	7.0	5.1	1.9	72	28	2.5	2.6
20	" 11	6.3	5.0	1.3	79	21	1.8	3.7
21	" 16	6.0	5.4	0.6	90	10	2.0	9.0
22	Mar. 1	5.6	3.8	1.8	70	30	2.0	2.3
23	" 8	6.4	6.0	0.4	93	7	2.1	13.2
24	" 15	6.4	5.0	1.4	78	22	2.2	3.5
25	" 22	6.1	3.6	2.5	60	40	2.7	1.5
26	Dec. 21	6.8	4.9	1.9	72	28	1.7	2.6
27	" 30	6.5	4.6	1.9	70	30	1.8	2.3
28	Jan. 6	6.9	4.9	2.0	71	29	1.6	2.4
29	" 6	6.2	4.8	1.4	77	23	1.7	3.3
30	" 6	6.1	4.3	1.8	70	30	2.1	2.3
31	Mar. 20	6.8	4.0	2.8	60	40	1.6	1.5
32	Apr. 3	5.7	4.5	1.2	79	21	2.1	3.7
33	" 25	5.0	3.5	1.5	70	30	2.5	2.3
34	May 1	6.1	4.5	1.6	74	26	2.0	2.8
35	" 4	5.5	4.0	1.5	72	28	2.2	2.6
Average.....		6.1	4.6	1.5	75	25	1.6	3.0
Jewett (6).....		6.13	4.82	1.31	79	21	2.0	

\* The protein quotient is the figure obtained by dividing the percentage of albumin by the percentage of globulin.

From Table I it is apparent that normal dogs may show some variation in the percentages of total protein, serum albumin, and serum globulin. These normal fluctuations are not large, however, when compared with those observed in the experimental conditions studied. Whereas the protein quotient in normal animals averages 3.0 and in most instances does not fall below 1.5, some of the animals with intoxication show at one period or another a quotient much below 1.0. We have taken these normal variations into account in that determinations of the normal percentages were made previous to the study of the experimental condition.

*Simple Obstruction for 5 Days. Toxic Death. Peritoneum Clean.*

*Dog 5.*—Shepherd, mongrel, female; weight 36 pounds.

Nov. 10. Simple obstruction in the middle of the small intestine by means of a complete section and an inversion of the ends.

Nov. 11 and 12. Dog is vomiting, and there is a steady loss of weight.

Nov. 13. Much thin, yellow vomitus. Temperature 102.8° F. Weight 33 pounds.

Nov. 15. Dog is severely intoxicated. Ether anesthesia. Killed.

*Autopsy.*—Thorax, lungs, and heart normal. Viscera show slight congestion. Peritoneum dry and clean. There are a few fibrinous adhesions about the site of obstruction. Intestinal tract below obstruction is collapsed and mucosa normal. No congestion or ulceration.

TABLE II.

*Dog 5. Simple Obstruction. Toxic Death.*

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Nov. 10	5.8	4.4	1.4	76	24	2.1	3.1	Operation. Non-coagulable nitrogen in blood 34 mg. Urea nitrogen 15 mg.*
" 12	6.6	3.4	3.2	52	48	2.6	1.1	
" 13	6.4	3.4	3.0	53	47	2.4	1.1	Non-coagulable nitrogen in blood 57 mg. Urea nitrogen 20 mg.
" 15	10.7	4.8	5.9	45	55	2.6	0.8	Killed.

\* In the tables non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.



The protocol of Dog 5 gives the clinical history of an animal succumbing to this type of obstruction. Constant vomiting and an acute terminal intoxication are the chief clinical features. From Table II it is clear that the progressive intoxication has registered definite changes in the blood proteins. 48 hours after the operation, the serum globulins had already increased 50 per cent. This increase, it would appear, is partly at the expense of the albumin fraction. And in spite of the progress of the intoxication, no further rise in globulins took place until the day of death, when this fraction constituted 55 per cent of the total proteins. The terminal increase of the total protein to double its initial value is also noteworthy. This is probably to be accounted for by the extreme dehydration which occurred during the last few days as a result of vomiting. From the protocol and table, it is clear that the percentage rise in globulins showed no tendency to parallel the increase in the severity of the intoxication.

TABLE III.

*Dog 6. Long Loop of Ileum. Volvulus of Loop.*

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Nov. 19								Operation.
" 22	5.9	4.2	1.7	70	30	1.7	2.3	Non-coagulable nitrogen in blood 30 mg. Urea nitrogen 14 mg.
" 24	5.1	2.9	2.2	57	43	1.4	1.3	
" 26	5.2	3.4	1.8	65	35	1.3	1.9	Non-coagulable nitrogen in blood 32 mg. Urea nitrogen 15 mg.
" 29	5.9	4.4	1.5	74	26	1.2	2.8	
Dec. 4								Found dead on 15th day.

*Long Loop of Ileum. Volvulus of Loop.*

*Dog 6.*—Black and white female; weight 20 $\frac{3}{4}$  pounds.

Nov. 19. A long circular loop of ileum was made by end to end anastomosis.

Nov. 20. Dog is well. Temperature 101.2°F. Weight 20 $\frac{1}{4}$  pounds.

Nov. 22. Dog is fairly well. Weight 19 $\frac{1}{2}$  pounds.

Nov. 24. No vomiting; eats.



Nov. 26. No vomiting. Dog very active and looks normal.

Nov. 27. Dog seems normal.

Dec. 4. Found dead in cage.

*Autopsy.*—Thorax negative. Spleen small and not engorged. Liver pale and not congested. Stomach, duodenum, and small intestine all pale and normal. Anastomosis in good condition. Loop is enormous and tense, dark, blackish green in color, and twisted about its pedicle. Mucosa intact, but grayish and probably necrotic. Peritoneal cavity shows slight injection, and contains 150 cc. of sticky fluid.

*Long Loop of Ileum. Slowly Progressing Intoxication. Proteose Injection.*

*Dog 2.*—Strong black and tan, bobtail, female; weight  $29\frac{1}{2}$  pounds.

Oct. 29. A long, circular loop of ileum was made by end to end anastomosis; about one-half of the small intestine was included.

Oct. 30. Dog is in good condition. Temperature  $102.5^{\circ}\text{F}$ . Weight  $29\frac{1}{2}$  pounds.

Nov. 1. There is much bile-stained vomitus below the cage. Temperature  $101.6^{\circ}\text{F}$ . Weight  $28\frac{1}{2}$  pounds.

Nov. 3. Dark, mucoid fluid below cage; partly vomitus and partly diarrhea. Pulse good.

Nov. 4. Dog much better; eats and looks well. No vomitus; no diarrhea. Temperature  $102.3^{\circ}\text{F}$ . Weight  $27\frac{1}{4}$  pounds.

Nov. 5. Fluid and bloody mucous diarrhea persists.

Nov. 8. Dog has slight nasal discharge. No diarrhea. Temperature  $102.2^{\circ}\text{F}$ . Weight  $25\frac{3}{4}$  pounds.

Nov. 9. Dog seems fairly well.

Nov. 11. Little diarrhea. No vomitus. Eats a little. Weight  $24\frac{1}{4}$  pounds.

Nov. 13. Dog is in good condition. Passed solid stool.

Nov. 15. Injection of pure proteose (pancreas I), alcohol precipitate 20 cc. Dog is resistant to proteose.

Nov. 16. Dog losing ground. Weight 24 pounds. Temperature  $102.4^{\circ}\text{F}$ . Nasal discharge but no frank distemper.

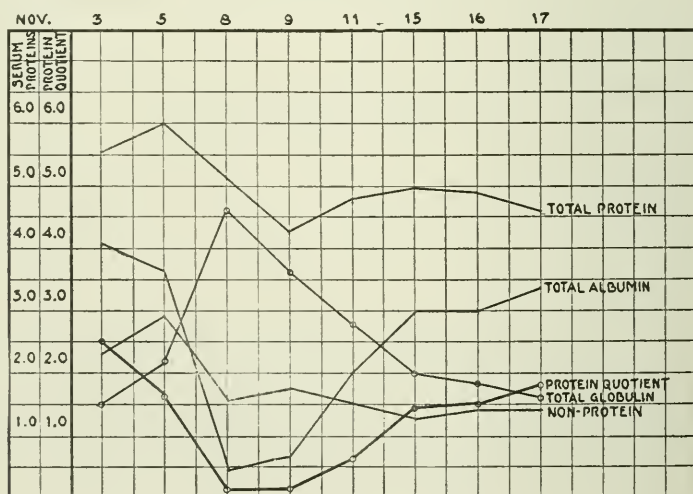
Nov. 17. Dog about the same. No vomiting. Strong and would live several days. Killed for blood.

*Autopsy.*—Peritoneum clean except for one small pus pocket close to the anastomosis in the loop. Peritoneal cavity contains a slight amount of turbid fluid. Stomach and small intestine are intact and pale. Anastomosis in ileum is clean and open. Liver is fatty. Bile pigments in urine and serum. The loop ends are joined, and the sutures are buried. No ulcer; mucosa has grown over the stitches. Wall of mucosa is double, but not thickened in spite of dilated loop. Mucosa everywhere pale and intact.

TABLE IV.

*Dog 2. Long Loop of Ileum. Proteose Injection.*

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Oct. 29								Operation.
Nov. 3	5.6	4.1	1.5	72	28	2.3	2.6	Non-coagulable nitrogen in blood 90 mg. Urea nitrogen 21 mg.
" 5	6.0	3.7	2.3	62	38	2.9	1.6	
" 8	5.1	0.5	4.6	10	90	1.5	0.11	Non-coagulable nitrogen in blood 53 mg. Urea nitrogen 20 mg.
" 9	4.3	0.65	3.6	12	88	1.7	0.13	
" 11	4.8	2.0	2.8	42	58	1.5	0.72	Non-coagulable nitrogen in blood 59 mg. Urea nitrogen 21 mg.
" 15	5.0	3.0	2.0	60	40	1.3	1.5	Non-coagulable nitrogen in blood 54 mg. Urea nitrogen 32 mg. Injection of proteose.
" 16	4.97	3.0	1.97	60	40	1.4	1.5	Non-coagulable nitrogen in blood 40 mg. Urea nitrogen 19 mg.
" 17	4.6	3.3	1.6	65	35	1.5	1.9	Killed.



TEXT-FIG. 1. Dog 2. Long loop of ileum. No peritonitis. Gradual rise in globulins.

The protocols of both of these experiments show that the intoxication in this type of closed intestinal loop is slowly progressing. Dog 6 lived 15 days, and Dog 2 was killed 19 days after the operation. The loop in these experiments was made by isolating a circular portion of the intestine and reestablishing the continuity of the rest of the intestine without obstructing the flow from the duodenum to the jejunum and ileum. The intoxication developed under these conditions is mild, and this explains the slight changes in the non-coagulable nitrogen of the blood.

Notwithstanding the mildness of the intoxication, both animals showed considerable alteration in the albumin-globulin ratio of the blood. Dog 6 is of great interest, because this animal showed no clinical symptoms of intoxication during any period of the experiment, and was found dead on the 15th day. The percentage of globulins, however, had increased 13 per cent on the 5th day (Table III), but on the day of death the globulins had returned almost to a normal level.

Dog 2 lived 3 weeks. This experiment is especially complete, since the blood examinations could be made over a longer period. In this instance, also, the toxic symptoms were not marked. The first definite rise in the globulins was noted only on the 7th day after the operation (Table IV). On the 10th day, the globulin fraction constituted 90 per cent of the total protein, which at this time showed only slight variation from its initial value. The reason for so marked a rise in the globulins of this animal is not clear. Since the total protein percentage on this day showed only little change, it must be assumed that the increase in globulins took place at the expense of the albumin fraction, which fell from an initial value of 4.1 per cent to 0.5 per cent. Following the maximum rise, the globulins began to decrease on the 13th day, gradually reaching 35 per cent on the day the animal was killed. The fluctuations in the various fractions are shown in Text-fig. 1.

Both experiments illustrate two points which deserve especial emphasis: first, the absence of any parallelism between the degree of intoxication and the percentage rise of globulins; and second, the tendency noted in several experiments for the globulin curve to return to its initial level. This would seem to indicate that the partition

of the serum protein may become normal when the cause or causes which alter the relative proportions are no longer at work.

*Long Loop of Jejunum. No Peritonitis.*

*Dog 15.*—Mongrel, black and white setter, male; weight 32½ pounds.

Feb. 1. A high loop of jejunum was made by end to end anastomosis.

Feb. 2. Dog rather quiet. Temperature 38.9°C.

Feb. 3. Some dark brown vomitus; pulse good. Temperature 38.4°C. Weight 32 pounds.

Feb. 4. No vomiting; lively.

Feb. 8. Dog in good condition. Temperature 38.3°C. Weight 30½ pounds.

Feb. 12. Condition the same. No vomiting.

Feb. 14. Dog is weak; pulse slow, but good volume. Temperature 38.3°C. Weight 27¾ pounds.

Feb. 16. Condition the same. Weight 26¾ pounds.

Feb. 17. Dog is losing ground. Weight 25½ pounds.

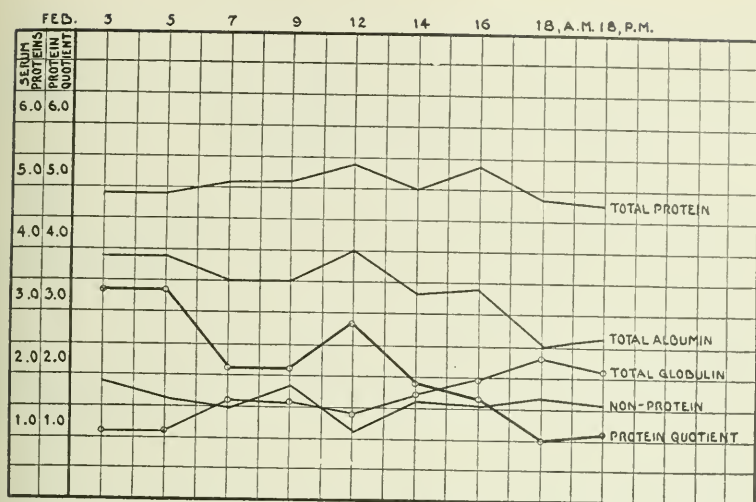
Feb. 18, 3.30 p.m. Dog is quite weak. Ether anesthesia. Killed.

*Autopsy.*—Thorax clean. Spleen small and fibrous. Stomach contains little fluid. Mucosa normal. Duodenum contains bright bile-stained fluid; mucosa normal. Anastomosis in good condition. Jejunum small and collapsed; mucosa normal. Peritoneal cavity moist and clean; contains a few cubic centimeters of

TABLE V.

*Dog 15. Long Loop of Jejunum. No Peritonitis.*

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Feb. 1								Operation.
" 3	4.9	3.8	1.1	77	23	1.8	3.3	
" 5	4.9	3.8	1.1	77	23	1.6	3.3	
" 7	5.1	3.5	1.6	68	32	1.5	2.1	Non-coagulable nitrogen in blood 29 mg.
" 9	5.1	3.5	1.6	68	32	1.7	2.1	
" 12	5.4	4.0	1.4	74	26	1.1	2.8	
" 14	5.0	3.3	1.7	66	34	1.7	1.9	
" 16	5.4	3.4	2.0	63	37	1.6	1.7	
" 18, a.m.	4.9	2.5	2.4	51	49	1.7	1.0	Non-coagulable nitrogen in blood 75 mg. Urea nitrogen 34 mg.
" 18, p.m.	4.8	2.6	2.2	54	46	1.6	1.1	Killed.



TEXT-FIG. 2. Dog 15. Long loop of jejunum. No peritonitis. Moderate rise in globulins.

blood-tinged fluid, probably due to twist of lower end of loop and omentum. Loop is huge and contains 750 cc. of dark brown syrupy fluid. Serosa normal. Mucosa intact everywhere. Few ecchymoses in washed mucosa, giving it a mottled brown and dull red color. Lower 4 inches of loop are deep red and acutely congested, due to partial twist of this end.

### *Long Loop of Jejunum. General Peritonitis.*

Dog 4.—Mongrel, male; weight 22 pounds.

Nov. 6. Long loop of jejunum made by section and inversion of ends; jejunum joined around the loop by means of end to end anastomosis between upper and lower end.

Nov. 8. Dog looked well. Temperature 102.4°F. Weight 21 pounds.

Nov. 9. Much vomiting. Weight 20½ pounds.

Nov. 10. Dog better. No vomiting.

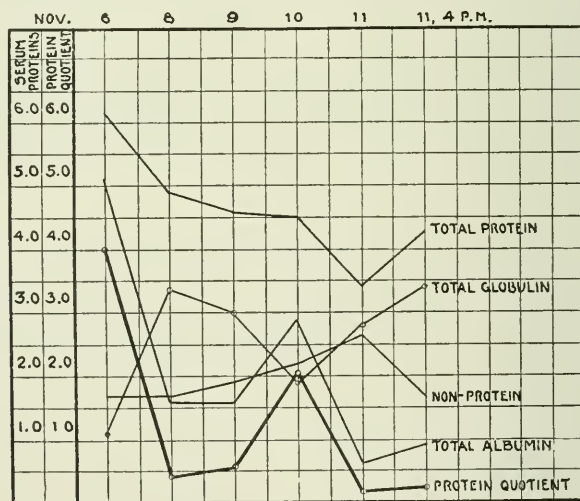
Nov. 11. Little vomiting. Temperature 100.2°F. Weight 21 pounds. 4 p.m. Dog is weak. Ether anesthesia. Killed.

*Autopsy.*—Thorax, heart, and lungs normal. Peritoneal cavity contains many isolated pockets of purulent exudate. A large, round worm is free in the peritoneal cavity, having escaped from the loop, which contains numerous similar worms. There are organized adhesions just below the end to end anastomosis, causing a sharp kink and probably complete obstruction. The loop contains 130 cc. of pale, slate-colored fluid with a strong odor. Mucosa of loop pale and intact. The peritonitis is probably of 1 or 2 days' duration.

TABLE VI.

*Dog 4. Long Loop of Jejunum. Obstruction. General Peritonitis.*

Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Nov. 6	6.3	5.1	1.2	80	20	1.7	4.0	Operation.
" 8	4.9	1.6	3.3	32	68	1.7	0.47	Non-coagulable nitrogen in blood 32 mg. Urea nitrogen 13 mg.
" 9	4.6	1.6	3.0	35	65	1.9	0.54	
" 10	—	—	—	—	—	—	—	
" 11	3.4	0.6	2.8	18	82	2.6	0.2	Non-coagulable nitrogen in blood 50 mg. Urea nitrogen 22 mg.
" 11, 4 p.m.	4.3	0.9	3.4	20	80	1.6	0.2	Killed.



TEXT-FIG. 3. Dog 4. Long loop of jejunum. General peritonitis. Precipitous rise in globulins.

Tables V and VI give the changes observed in the serum proteins of animals with closed loops of the jejunum. In these dogs, also, the loops were so made that there was unobstructed flow through the duodenum. Dog 15 lived 18 days (Text-fig. 2), and showed through-



out only a mild intoxication. The percentage of globulins rose only moderately on the 6th day, and on the day the animal was killed the globulins constituted 46 per cent of the total protein. At autopsy the loop was found much distended with fluid, but the peritoneal cavity was moist and clean.

In Dog 4 the same experimental condition was produced, but this experiment illustrates one of the complications which may arise in animals with closed loops. This dog developed a peritonitis as a result of leakage from the loop, and was killed 5 days after the operation. 2 days after the loop had been isolated, the percentage of globulins had already risen from 20 to 68 per cent. The percentage of total protein fell, and, as will be seen from Text-fig. 3, the rise in globulins occurred largely at the expense of the albumin fraction.

On the day the animal was killed, the globulins constituted 82 per cent of the total protein,—a complete inversion of the normal ratio. At autopsy 130 cc. of fluid were found in the peritoneal cavity, the peritonitis appearing to be of 1 or 2 days' duration. But according to the other experiments with uncomplicated closed loops, such a rapid and marked rise in the globulin fraction is not the usual occurrence. This would indicate that the leakage of toxic material with its rapid absorption may well have commenced soon after the operation. This observation together with others furnishes good evidence that a rapid absorption of toxic exudate may give rise to an early and large increase in the percentage of serum globulin.

#### *The Effect of Intravenous Injections of Proteose upon the Albumin-Globulin Ratio.*

The immunity to proteose injections shown by dogs with long-standing closed intestinal loops indicates that the presence of a closed loop causes a chronic proteose intoxication, which gives the animal a certain degree of immunity against these toxic substances. In order to determine whether the rise in serum globulins observed in these loop experiments was associated with the development of this tolerance, the observations recorded in Table VII were made. Three normal dogs were injected intravenously with small sublethal doses of pure proteose. Injections were given at weekly intervals

TABLE VII.

*Proteose Injections. Normal Albumin-Globulin Ratio.*

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	per cent	per cent	per cent	per cent	per cent		
7	Nov. 29	6.3	5.2	1.1	83	17	1.7	4.8	Injection of 10 cc. of cat loop proteose. Vomiting and elevation of temperature.
	Dec. 1	5.6	4.4	1.2	78	22	1.5	3.5	Dog lively and in good condition.
	" 6	6.1	4.1	2.0	67	33	1.9	2.0	Injection of 10 cc. of cat loop proteose. Dog sick; vomited.
	" 20*	5.8	4.5	1.3	77	23	1.9	3.3	Two proteose injections, one on Dec. 13 and another on Dec. 20.
8	Nov. 29	6.2	5.0	1.2	80	20	1.7	4.0	Injection of 10 cc. of peritoneal exudate proteose. Slight elevation of temperature.
	Dec. 1	5.2	3.1	2.1	59	41	1.4	1.4	
	" 6	6.6	5.2	1.4	78	22	1.7	3.5	Injection of 15 cc. of peritoneal exudate proteose. Vomiting, slight diarrhea.
	" 9	5.8	4.4	1.4	75	25	1.6	3.0	
	" 13	5.9	4.6	1.3	79	21	2.0	3.7	Injection of 15 cc. of loop fluid. Elevation of temperature; slight vomiting.
	" 20	6.0	4.4	1.6	73	27	2.0	2.7	Injection of 20 cc. of peritoneal exudate. Elevation of temperature; slight intoxication.
9	Jan. 5	5.8	3.9	1.9	67	33	1.7	2.0	Injection of 25 cc. of proteose on Dec. 27 and of 35 cc. on Jan. 5. Rapid recovery. Slight intoxication.
	Nov. 29	6.4	5.3	1.1	82	18	1.6	4.5	Injection of 6 cc. of loop proteose. Slight vomiting.
	" 29	5.9	5.1	0.8	86	14	1.4	6.1	Specimen obtained 2 hrs. after injection.
	Dec. 6	6.5	5.3	1.2	81	19	1.4	4.3	Injection of 10 cc. of loop proteose. Slight vomiting.

\* The specimen was obtained immediately after the injection.

TABLE VII—*Continued.*

No. of dog.	Date.	Total protein. <i>per cent</i>	Total albumin. <i>per cent</i>	Total globulin. <i>per cent</i>	Albumin of total protein. <i>per cent</i>	Globulin of total protein. <i>per cent</i>	Non-protein constituents. <i>per cent</i>	Protein quotient.	Remarks.
9	Dec. 9	6.0	4.1	1.9	68	32	2.3	2.1	
	" 13	5.6	4.2	1.4	74	26	1.8	2.8	Injection of 15 cc. of loop proteose. Elevation of temperature and vomiting.
	" 20	6.2	5.0	1.2	80	20	1.7	4.0	Injection of 20 cc. of loop proteose. Slight intoxication.
	Jan. 5	6.1	5.0	1.1	82	18	1.7	4.5	Injection of 25 cc. of proteose on Dec. 27 and 35 cc. on Jan. 5. Slight intoxication.
36	Dec. 20	6.6	5.4	1.2	81	19	1.5	4.2	Injection of 10 cc. of proteose from human peritoneal exudate; subsequent injections of 15 cc. and 25 cc. on Dec. 27 and Jan. 5. Very slight intoxication.
37	Jan. 5	6.9	4.9	2.0	71	29	1.4	2.4	
	Apr. 6	5.3	3.0	2.3	60	40	2.1	1.5	Drained loop made on Dec. 23. Dog in good condition until Apr. 6. when 27½ cc. of loop fluid proteose were injected. Vomiting, diarrhea, and intoxication.
	" 7	5.6	2.8	2.8	50	50	2.5	1.0	Died 24 hrs. later. Specimen obtained at autopsy.

and analyses of the blood were made before each injection. The proteose was prepared either from loop fluid or from peritoneal exudates. After the second injection the animals responded well to larger doses, and showed only slight signs of intoxication.

It is clear from Table VII that dogs injected with small doses of proteose do not show any alteration in the albumin-globulin ratio of the blood serum. The occasional slight rise which has been observed is within the limit of the variations noted in normal dogs. It would appear, therefore, that although the animals developed a tolerance for increasing doses of proteose, the globulin content remained unchanged. It should be emphasized that this is a slowly devel-

TABLE VIII.

*The Effect of Inflammatory Conditions (Peritonitis, Pleurisy, and Pancreatitis) upon the Albumin-Globulin Ratio.*

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
38	Feb. 23	7.8	3.3	4.5	42	58	2.8	0.7	<i>Peritonitis.</i> Gastrojejunostomy done on Feb. 18. Died 5 days later of general peritonitis.* Specimen obtained on day of death.
39	Apr. 21	5.3	1.0	4.3	23	77	3.9	0.3	<i>Pleurisy.</i> Injection of 1 cc. of turpentine and 5 cc. of aleuronat into right pleural cavity on Apr. 7. Second injection of 1 cc. of turpentine on Apr. 19.
									<i>Autopsy.</i> —Thick fibrinopurulent exudate over lungs.
16	Mar. 20	5.2	2.7	2.5	52	48	2.4	1.1	<i>Pleurisy.</i> Mar. 13. Injection of 1 cc. of turpentine and 5 cc. of 1 per cent aleuronat into right pleura. Mar. 17. Injection of $\frac{3}{4}$ cc. of turpentine and 5 cc. of aleuronat. Aspiration of 50 cc. of bloody fluid on Mar. 18 and 10 cc. on Mar. 20.
									<i>Autopsy.</i> —Mar. 23. Purulent and fibrinous exudate in right pleura. About 40 cc. of blood-tinged fluid obtained.
40	Apr. 21	5.6	3.0	2.6	53	47	3.2	1.1	<i>Pancreatitis.</i> Apr. 20. 10 cc. of fresh dog bile injected into pancreatic duct.
									<i>Autopsy.</i> —Apr. 21. Little fluid in peritoneal cavity; pancreas large, edematous; moderate hemorrhage in stroma; slight amount of fat necrosis. Not an extreme injury of gland.

\* A more marked increase in the percentage of globulins associated with the development of a peritonitis was observed in Dog 4.

TABLE VIII—*Continued.*

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	per cent	per cent	per cent	per cent	per cent		
41	Apr. 26	5.4	1.0	4.4	19	81	2.2	0.2	<i>Turpentine abscess and pancreatitis.</i> Apr. 20. Injection of 2 cc. of turpentine into left thorax. Apr. 26. Injection of 10 cc. of dog bile and glycocholic acid. Killed 7 hrs. later. <i>Autopsy.</i> —Abscesses in tissues. Pancreas large, dark purple, with extreme edema and fat necrosis.

oping tolerance for proteose which is produced under these experimental conditions.

*The Effect of Peritonitis, Pancreatitis, and Pleurisy upon the Albumin-Globulin Ratio.*

Table VIII contains some observations upon various inflammatory conditions in which the partition of the protein fractions was studied. In Dog 38 the peritonitis resulted from leakage following gastro-jejunosomy. The high percentage of globulins noted in this experiment is in harmony with similar observations (Table VI).

Dogs with sterile pleurisy produced by the injection of turpentine and aleuronat also show a high globulin content in the serum. In these experiments the presence of bacteria in the pus formation was excluded by obtaining sterile cultures from the exudate. Notwithstanding this, however, a considerable increase in globulins occurred. This observation supplements the results of previous experiments. We have shown in a previous paper (1) that bacterial toxins obtained from bacterial growths by Berkefeld filtration may give rise to just as marked an alteration in the ratio as the injection of living and killed cultures of the organisms themselves.

The experiments with acute hemorrhagic pancreatitis produced by the injection of dog's bile into the pancreatic duct further emphasize this point. Dog 41 is of especial interest, because in this animal the pancreatitis was complicated by a turpentine abscess. This animal showed a complete inversion of the albumin-globulin ratio, the globulins constituting 81 per cent of the total protein.

TABLE IX.

*Serum Proteins in Dogs with Distemper.*

No. of dog.	Date.	Total protein. per cent	Total albumin. per cent	Total globulin. per cent	Albumin of total protein. per cent	Globulin of total protein. per cent	Non-protein constituents. per cent	Protein quotient.	Remarks.
42	Nov. 8	7.0	5.6	1.4	80	20	1.4	4.0	Sept. 22. Operation, drained loop of duodenum and jejunum. Animal in good condition until Oct. 21. Developed distemper, which was quite severe on Nov. 8. <i>Autopsy.</i> —Large patches of bronchopneumonia.
43	Oct. 30	5.9	3.0	2.9	51	49	1.9	1.0	Dog in poor condition for a week before examination of blood. Profuse nasal discharge. <i>Autopsy.</i> —Lungs show only a few hemorrhagic specks.
44	Nov. 4	5.9	4.3	1.6	73	27	1.7	2.7	Slight grade of distemper.
45	Dec. 23	6.8	4.1	2.7	60	40	2.3	1.5	Acute infection. Distemper. <i>Autopsy.</i> —Hemorrhagic specks and patches in lungs.
46	Jan. 5	6.7	4.3	2.4	64	36	1.6	1.7	Died on Jan. 17 of distemper.
47*	Mar. 22	4.2	2.4	1.8	57	43	2.0	1.3	<i>Autopsy.</i> —Patches in both lungs.

\* Pup.

During the course of the experiments a few other conditions were studied which deserve brief mention. An opportunity was afforded to study a few animals with distemper (Table IX). With the infection of the respiratory passages these animals may at times show



well marked signs of intoxication. Of six dogs studied only two showed any considerable alteration in the ratio. Two dogs showed a moderate change, and in two animals the albumin and globulin percentages were normal. From these few observations no definite conclusion is permissible, but it would appear that even where the globulin rise in this infection does occur, the percentage increase is not nearly so marked as that noted in some of the loop animals with complications or in those with abscess formation and tissue destruction.

TABLE X.

*Serum Proteins in Dogs with Eck Fistula.*

No. of dog.	Date.	Total protein.	Total albumin.	Total globulin.	Albumin of total protein.	Globulin of total protein.	Non-protein constituents.	Protein quotient.	Remarks.
		per cent	per cent	per cent	per cent	per cent	per cent		
48	Nov. 16	5.9	4.0	1.9	69	31	1.4	2.2	Sept. 13. Operation. Normal condition.
49	Dec. 28	5.7	4.2	1.5	74	26	1.6	2.8	Sept. 14. Operation. In excellent condition until Nov. 16. Moderate distemper and loss in weight. Poor appetite and gradual downward course. Definite intoxication on Dec. 28. Convulsions followed by death on Dec. 29.
50	Nov. 22	7.3	4.2	3.1	58	42	1.5	1.3	<i>Autopsy.</i> —Eck fistula perfect. Oct. 12. Operation. In excellent condition until Oct. 21. Development of distemper. Nasal discharge and cough. Condition on Nov. 22 not so good. Nose ulcerated.
51	Apr. 5	5.5	3.9	1.6	71	29	1.7	2.4	Feb. 22. Operation. In excellent condition on Apr. 5.
52	" 18	4.6	2.2	2.4	47	53	2.3	0.9	Mar. 10. Operation. Splenectomy on Mar. 23. Moderate loss in weight. Some vomiting and poor appetite for 10 days before date of blood examination.

Similarly, the blood of a number of Eck fistula dogs (Table X) was studied. Only one of the animals observed presented definite symptoms of intoxication at the time the protein partition was determined, and in this instance it was found normal. Another animal (Dog 52), on the other hand, showed a definite rise in globulins, but this animal had been in poor condition for 10 days prior to the blood examination. The low protein content obtained in this animal on this date is in keeping with the loss of weight and the impaired nutrition. Dog 50 also showed an increased globulin content, but this animal had developed distemper a month previously, and still had some signs of infection when the blood analysis was made.

#### DISCUSSION.

A satisfactory explanation of our experimental observations would require a knowledge concerning the origin and chemical relation of the various protein fractions, which we do not possess at present. It is permissible, however, to discuss several points on the basis of the data available. According to the work of some observers, the increase in globulins might be regarded as one of the phenomena which attend the development of the immunity or tolerance gradually acquired by an animal with long-standing obstruction or that following the repeated injections of proteose. Moll (7) has given some experimental evidence in support of such a view by showing that the formation of precipitins in the sera of animals injected with foreign protein parallels in a measure the rise in the globulins which occurs in them. Our experiments, however, do not give support to this interpretation. As already pointed out, we were unable to demonstrate that the development of resistance by an animal is accompanied by a heaping up of globulins in the blood. It will be recalled that following the repeated proteose injections in immunizing doses no increase in the serum globulins took place; whereas, in the loop animals in which this was a striking feature we were unable to show that the globulin increase was in any way related to periods of clinical improvement, or that a fall was associated with the development of a more severe intoxication.

It would seem more probable that the alteration in the normal

partition of albumins and globulins, and the complete inversion of the ratio following the rapid absorption of a toxic exudate is due to some fundamental change in metabolism. That such a metabolic upset occurs in the intoxications studied has been demonstrated by one of us (2). It has been shown that the exciting cause in some of these intoxications is the absorption of a toxic substance resulting from local tissue disintegration, or an upset in the delicate protein equilibrium. These poisons so formed are widely distributed in the body, and cause much protein injury. One result of this extensive destruction of body protein is the accumulation of non-coagulable nitrogenous substances in the blood, and an increased elimination of nitrogen immediately following the injury. It is, therefore, not unreasonable to assume that such a marked metabolic disturbance may also register a change in the coagulable proteins of the blood.

Still more difficult to explain, however, is the rapidity with which the inversion of the ratio is produced, and the increase of the globulins at the expense of the albumin fraction. From a theoretical viewpoint, a heaping up of blood globulins might be the result of a more rapid formation or a less rapid destruction of this substance or, lastly, of its diminished utilization. From what has already been said, it would seem that catabolism is especially active in these intoxications, and that anabolism is more or less at a low ebb. On the basis of the experiments now available, it is not possible to state with certainty which of the metabolic phases is more responsible for the changes observed. According to the work of Moll (8), the rise in the globulin content may be explained as the result of a more rapid conversion of albumin into globulin due to the accelerated metabolism; whereas the observations of Cervello (9) suggest the explanation that a heaping up of globulins may result from the diminished utilization of them by the body tissues.

It is worth while keeping two facts in mind. With an acute and profound intoxication of almost any type, we usually observe more or less increase of the globulin fraction at the expense of the albumin portion. With a slowly induced tolerance or immunity to a proteose or similar poison following repeated small doses of the poison, we usually observe little change, if any, in the globulin-albumin ratio. There is no relation between the tolerance or immunity developed

and the upset in the globulin-albumin fractions. There does seem to be a relation between the shock and intoxication caused by the poison and the globulin increase: in general, the more profound and acute the intoxication, the more does the globulin increase at the expense of the albumin, even to an inversion of the normal ratio.

These severe intoxications show a remarkable increase in the elimination of urinary nitrogen above the normal base-line level (Whipple, Cooke). There is strong evidence for profound tissue injury with an upset in the delicate protein balance of the body cells. May we assume that the change in the blood proteins is primary and dependent upon the direct action of the poison, or may we assume that the blood protein changes, like the blood non-protein changes, are dependent upon a primary cell protein reaction? More experimental work is required to answer these and many other questions concerning the blood proteins about which our knowledge is incomplete.

#### CONCLUSIONS.

The intoxication which develops as the result of a simple obstruction or a closed intestinal loop is accompanied by definite changes in the coagulable proteins of the blood serum. These changes consist essentially in an alteration in the normal albumin-globulin ratio; the globulin fraction is greatly increased and at times the normal relation of the two fractions may show a complete inversion.

The increase in the globulin content of the blood serum is most marked in the animals which show some of the complications met with in loop animals,—rupture of the loop and peritonitis. In the latter conditions especially, the globulin increase is rapid and large. We believe this reaction to be of diagnostic value in acute infections attended by the sudden liberation and absorption of a toxic exudate.

Infections and intoxications produced by inflammatory irritants are also accompanied by a rise in the blood globulins. This observation suggests that tissue disintegration with absorption of toxic products is responsible for the changes noted, and that bacterial invasion is important only in as far as it gives rise to toxic substances.

Animals which have developed a tolerance to proteose intoxication following the periodic injection of small doses of proteose do not



show a globulin increase. These experiments do not support the view that the rise in globulins observed in these experimental conditions is an expression of a resistance or tolerance developed by the animal.

From the experimental evidence it seems more probable that the alteration in the partition of the blood protein fractions is one of the results of the metabolic disturbance which has been shown to occur in these conditions.

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ON THE NYMPH AND PROSOPON OF THE TSUTSUGA-  
MUSHI, LEPTOTROMBIDIUM AKAMUSHI, N. SP.  
(TROMBIDIUM AKAMUSHI BRUMPT),  
CARRIER OF THE TSUTSU-  
GAMUSHI DISEASE.

By MATARO NAGAYO, M.D., YONEJI MIYAGAWA, M.D., TOKUSHIRO  
MITAMURA, M.D., AND ARAO IMAMURA, M.D.

(*From the Imperial Institute for Infectious Diseases, Tokyo, Japan.*)

PLATES 24 TO 27.

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INTRODUCTION.

Tsutsugamushi or kedani disease is an acute exanthematous infectious disease, which at present occurs only in the northern coast districts of Japan; *i.e.*, in Niigata ken, Akita ken, and Yamagata ken. Recently it has also been found to exist in Formosa (1). The mortality of the disease varies, according to the district and year, between 20 and 50 per cent. It closely resembles Rocky Mountain spotted fever, but, as Ashburn and Craig (2) have pointed out, it possesses some distinct features. Among previous investigators of the disease may be mentioned Baelz, Kawakami, Tanaka, Kitasato, Ogata, Asakawa, Kitajima, Miyajima, Hayashi, Kawamura, Arima, Sakai, and Hattori, who have studied the etiological, pathological, and clinical features. The specific cause of the disease has not yet been established with certainty. Last year we undertook to investigate the disease, and especially the question as to its cause and means of transmission. As regards the specific germ we expressed the opinion that certain piroplasma-like forms discovered by us, which occur in the spleen, lymphatic glands, and in the blood, but not in the red corpuscles, and which may belong to the Sporozoa, may be the cause (3). We shall not describe them here in detail, but shall confine our report to the carrier of the disease, a species of Trombidium.

That tsutsugamushi disease is transmitted to man by a diminutive mite, is now established. Baelz (4), to whom we owe the first scientific description of the disease, was at first not inclined to admit this fact, but it was undeniably proven by later investigators through clinical observation and experiments on animals. This mite is called in Japan tsutsugamushi (disease mite), akamushi (red mite), kedani (hairy mite), or shimamushi (island mite), and is similar to the European harvest-bug, *Leptus autumnalis* (Baelz). Tanaka (5) was the first to describe it in detail. The specific determination was first undertaken in 1910 by Brumpt (6), who considered it to be a larva of a new trombidium, which he called *Trombidium akamushi*, apparently, however, without finding its nymph and prosopon. Recently Kawamura and Komagata (7) have published a detailed description of the external and internal structures of this mite. The studies mentioned above refer only to the larva of a hitherto unknown trombidium, *Trombidium akamushi* Brumpt, which attacks warm-blooded animals as well as man. The nymph and the prosopon of the tsutsugamushi, however, do not bite warm-blooded animals and have not yet been detected with certainty. Several authors claim to have discovered or bred the nymph or the prosopon, but their reports are not convincing. Some trombidium larvæ are well known in Europe and America under the names *Leptus autumnalis*, *Leptus irritans*, *Leptus americanus*, harvest-bug, redbug, chigoe, etc. They also bite human beings and are more or less closely related to the tsutsugamushi; the other larvæ, however, never cause so serious a disease as the latter. That the investigation of this mite has so far been neglected is chiefly due to the danger of the bite which causes the disease, and to which the investigators are exposed while working in the infected region.

Since beginning the present investigation, from November, 1915, until August, 1916, we have frequently been in the infected districts from 2 days to 1 week every month. In summer, when there is danger of being bitten by the mite, we dressed in such a way as not to expose any part of the body except the eyes; and we rubbed the body with some mite-killing agent, such as phenol, sulphur ointment, or insect powder. At other seasons, when there is usually no danger of being bitten, we could work without special precautions. Of the endemic regions in Japan we selected as a place for research the environs of Yachi on the banks of the Mogami river in Yamagata ken, which for the last 3 years has been known as an endemic district. We visited the district near Yuzawa in Akita ken once only. In the suburbs of Tokyo, where the tsutsugamushi is not found, and consequently the disease does not occur, we have on several occasions collected various trombidia. After investigations extending

over 1 year, we are now in a position to report on the nymph and the prosopon of the tsutsugamushi. We were often misled by the circumstance that in the endemic district various kinds of trombidia are found, which in contrast to the prosopon of the tsutsugamushi are more striking in size and color, and therefore more easily found than the prosopon of the tsutsugamushi. At first we mistook a particular species of trombidium for the parent of the tsutsugamushi, as other authors have also done both before and after us. Subsequently we found that there occur a number of species of trombidium in the endemic region and we have been able to isolate with certainty at least five species, which we have called provisionally Trombidia A, B, C, D, and E (8).

Trombidia A, B, C, and D lay their eggs in culture soil, and we were able to raise larvæ from the eggs. With Trombidium B we succeeded in raising nymphs from the larvæ. We also found the nymphs of Trombidia A, B, and C in nature. We shall not go into detail about the various species of trombidium, but merely point out that Trombidium B may be regarded as a variation of *Trombidium fuliginosum* Herm, and that Trombidium D cannot be distinguished, at least morphologically, from the European *Trombidium holosericeum* Linné. Trombidia A, C, and E are perhaps new species.

The larva of Trombidium B somewhat resembles the tsutsugamushi, and at first we believed them to be identical. In raising the eggs of Trombidium D we watched their development closely, because we thought that we might be able to raise tsutsugamushi from them, as *Trombidium holosericeum* has generally been considered to be the parent of *Leptus autumnalis* (Mégnin (9) ), and the latter is so closely related to the tsutsugamushi that it may only be a variation of it, and as *Trombidium holosericeum* corresponds to Trombidium D. But since the larvæ obtained differed in essentials from tsutsugamushi and from *Leptus autumnalis*, we were forced to the conclusion, that *Trombidium holosericeum* Linné, is not the parent of *Leptus autumnalis*, as some authors have observed.<sup>1</sup> We believe that we have found the real parent of the tsutsugamushi, and we wish to report

<sup>1</sup> See Nagayo, M., Miyagawa, Y., Mitamura, T., and Imamura, A., Is *Trombidium holosericeum* the Parent of *Leptus autumnalis*, *J. Exp. Med.*, 1917, xxv, 273.

here on the nymph and the prosopon of the tsutsugamushi, which have hitherto remained unknown.

*The Nymph of the Tsutsugamushi.*

On April 30 and May 2, and 3, 1916, we received from Okiage and Nakagawara numbers of tsutsugamushi-carrying field mice. The lobes of their ears, which were thickly covered with the mites, were cut off and put into a glass dish. The mites then gradually loosened their hold and left the host, the fully fed ones sooner than the others. The mites were put in glass bottles, test-tubes, and glass dishes, which had been provided with moistened soil from the endemic districts, thoroughly sterilized by heat. On June 8, about 40 days later, three nymphs were found in one bottle, and later more (twenty-one in all) in the other vessels. Hayashi (10) and Kitajima and Miyajima (11) claim to have bred nymphs artificially before, but they do not describe the nymphs adequately, so that we cannot compare ours with theirs. We experienced no great difficulty in the culture of the nymphs. It is necessary to protect the soil from drying and rotting during the period of the experiment. The nymphs show great motility and like to hide in the ground. The second cultivation experiment, which we undertook with the larvæ taken from field mice caught on July 19, was likewise successful, and took a much shorter time than the first. After 10 days, on July 29, we found small nymphs crawling about, upon and in the soil of the vessels. The total number of nymphs produced amounted this time to over 80. They were a little smaller than those of the former breed, so it may be presumed that in the first experiment the nymphs were already some days old when they were discovered.

On July 30 we received two field mice from Nakagawara and for the third time we used the numerous attached tsutsugamushi for breeding the nymphs. For the purpose of studying the evolution of the pupa we distributed the well fed larvæ in eight breeding vessels, each of which contained 20 to 30 larvæ. After 3 full days, *i.e.*, on August 2, the first vessel was examined, and the others one by one on each succeeding day. This time the nymphs came out after 8 days, on August 7. The metamorphosis of the larva into the nymph



proceeds, though subject to slight variation, in general in the same manner as that described by Henking (12) for *Trombidium fuliginosum*. On the 4th day we already found the nymphophane. The oval immovable pupa is in every case distinguished by the formation of the so called *Apoderma* of Henking (the *Zwischenhaut* of Claparède (13)), lying under the larval cuticle. This is nearly transparent and supplied with single granule-holding round cells, which may, however, occasionally form groups (*Haemamoeben* of Claparède). Below this skin is the thin nymphal cuticle. The new extremities are already budding out and are likewise enveloped by the apoderm. There are now four pairs, the fourth pair being a new formation, as Henking has already described in *Trombidium fuliginosum*. The original three pairs of legs and the mouth apparatus have already undergone complete histolysis, so that nothing is visible under the original larval skin. The legs break off easily and are always outstretched, they never take the bent position, which is often found in dead larvæ. The original mouth apparatus is pushed somewhat forward and is easily detached from the body of the nymphophane. In the course of the following days the newly formed legs, of which the first pair is the best developed, grow longer and show a distinct arrangement of the parts and claws. They are directed obliquely backwards and towards the middle, so that the distal ends of each pair touch in the median line.

We have less to say of the development of the mouth apparatus. On the 4th day a small hemispherical protuberance is seen under the original mouth part of the larva, completely separated from the latter by the apoderm. Within this prominence one sees the anlage of the mouth parts; on the following days one can gradually distinguish the palpi, directed ventrally and backwards, and the mandibles between them. On the dorsal side of the head a peculiar spur-like structure is seen, with its point directed anteriorly. It projects from the chitinous skin of the nymph and reaches through the apoderm almost to the original larval skin. Henking does not mention this structure in *Trombidium fuliginosum*; it is probably an organ of the nymph to enable it to come out of the larval cuticle. In an 8 day old nymphophane we had excellent proof of this; we saw how the chitinous cuticle of the larva was pierced and torn by

this spur. The apoderm meanwhile increases in thickness and becomes provided with numerous acuminate excrescences. Later it grows thinner again, often becomes folded, and shows irregularly stratified lamellar structures. Through this apoderm the underlying newly formed hairs of the nymph are seen. The round cells, fairly numerous at one time, gradually disappear again. Shortly before the nymph emerges, the apoderm undergoes granular degeneration. Thus the evolution of the nymph is completed within a relatively short time, so that on the 9th day the majority of the new nymphs are found in or upon the soil of the receptacle.

### *Morphology of the Nymph.*

*Size and Form.*—The nymph is a small, rather long, oval mite with eight legs, and shows between the cephalothoracic region and the abdomen a distinct constriction, especially conspicuous during life. The thorax and abdomen are correspondingly rounded at the sides, and can easily be distinguished. The length of the body from the points of the mandibles is 0.342 to 0.644 mm.; the maximum width of the cephalothoracic region is 0.168 to 0.280 mm.; and that of the abdomen 0.198 to 0.324 mm.

TABLE I.

*Measurements of the Legs of a Nymph 0.612 Mm. in Length.*

	Total length.	Joint.					
		1	2	3	4	5	6
	mm.	mm.	mm.	mm.	mm.	mm.	mm.
1st pair.							
Length.....	0.432	0.028	0.060	0.060	0.072	0.088	0.124
Breadth.....		0.036	0.036	0.046	0.052	0.060	0.060
2nd pair.							
Length.....	0.236	0.023	0.028	0.028	0.040	0.040	0.068
Breadth.....		0.036	0.040	0.036	0.034	0.028	0.024
3rd pair.							
Length.....	0.208	0.024	0.024	0.024	0.028	0.044	0.064
Breadth.....		0.040	0.028	0.032	0.028	0.024	0.020
4th pair.							
Length.....	0.284	0.040	0.040	0.028	0.044	0.052	0.080
Breadth.....		0.036	0.032	0.032	0.028	0.028	0.024



*Color.*—The body is grayish white or pale yellow, while the legs are colorless. The contents of the stomach shining through the body give it the yellow color. The hairs and epidermis are colorless.

*Legs.*—There are four pairs of legs. The first pair is the longest, the fourth pair coming next, then the second and third (Table I).

The anterior pair is by far the longest; the segments, in contrast to the other legs, become longer and broader towards the distal end. Each tarsus is provided with two claws but has no clinging hairs. The claws of the anterior pair are much shorter (length 0.016 mm.) and less curved than those of the other legs (0.022 mm.).

*Epimeres.*—The epimeres of the first and second pairs of legs of the same side lie close together, as do those of the third and fourth pairs, though nowhere grown together, so that they form on each side an anterior and a posterior group. The surface of the epimeres is finely punctated. The first pair of epimeres extends distally for about one-third of their length over the edge of the body.

*Measurements of the Epimeres.*

	Leg.			
	1	2	3	4
	mm.	mm.	mm.	mm.
Length.....	0.080	0.060	0.068	0.076
Width.....	0.044	0.048	0.052	0.048

Distance between the anterior and posterior groups.....	mm.	0.048
Transverse distance between the pairs of the anterior groups.....		0.020
“ “ “ “ “ “ “ “ posterior “.....		0.060

*Tracheal and Stigmatal Plate.*—We have not yet found with certainty the tracheal openings which are distinctly visible in the larvae on the first pair of the epimeres. At least the scaly chitinous structure surrounding the tracheal openings, Henking's *Stigmenschutzapparat*, which is distinguished in the nymphs and prosopa of other trombidia, is not distinctly developed in ours.

*Genital Opening and Anus.*—The genital opening is oval and is situated in the median line of the ventral side of the abdomen, behind the fourth epimeres. The anus opens further back.

	Genital opening. mm.	Anus. mm.
Length (sagittal diameter).....	0.072	0.056
Width (cross diameter).....	0.048	0.040

The genital opening is surrounded on either side by a raised portion, provided with two flat oval plates, sucking discs.

*Mouth Apparatus.*—The palpus consists of four joints besides the basal part. The second is the largest in size. The terminal joint is provided with a claw (length 0.024 mm.) and a longer appendage, the thumb (length 0.036 mm.). This appendage is not club-shaped, as is usual with trombidia, but tapers towards the end and is covered with numerous pinnate hairs. If we regard this appendage as an independent joint of the palpus, as is done by Banks (14), Fiebiger, and others, we may count five joints in the latter.

*Size of the Palpi.*

	Joint.			
	1	2	3	4
	mm.	mm.	mm.	mm.
Length.....	0.016	0.048	0.036	0.034
Width (greatest).....	0.020	0.044	0.032	0.020

*Mandibles.*—The mandible, which averages 0.112 mm. in length has at the front a sharp, dorsally curved hook (length 0.052 mm., width 0.020 mm.), with fine teeth on the concave edge. Both mandibles are imbedded in a boat-shaped area, which gradually tapers towards the front and is pilose. The mandibles are covered at the posterior part by a corrugated epithelium, an elongation of the dorsum of the body, the epistome. A little in front of this epistome there is one hair, feathered and pointed, 0.0334 mm. long, in the median line between both mandibles.

*Scutum.*—The scutum consists of two symmetrical circular parts (the distance between them 0.020 mm.), each bearing a long hair (length 0.100 mm.), and a middle part connecting the two, which extends behind into a short process and forward into a hairless chitinous crista reaching to the end of the epistome.

*Eyes.*—The eyes are not easily visible. They are sessile and situated close to the pseudostigma of the scutum. The eye contains

fine reddish granules, but is on the whole rudimentary, compared with that of the larva, and is not provided with clearly developed lenses.

*Hairs*.—The paired tactile hairs of the scutum have already been mentioned. The entire body of the nymph is thickly covered with hairs. The most striking are those on the back of the abdominal segment, which are longer than the others (0.056 to 0.080 mm.), and have a rather thick shaft and club-shaped end. The surface of the shaft is covered with innumerable small pinnate hairs. The clubbed shape of the end varies in different individuals, and probably depends on the nourishment and state of development of the nymph. The length of the hair, too, shows individual variations. These hairs seem to be peculiar to the nymph of the *tsutsugamushi*, and have so far been found besides only in *Trombidium holosericeum* among our trombidium species. They differ from the latter in that they are colorless, while the club-shaped hairs of *Trombidium holosericeum* are red. They arise from distinctly developed hair follicles. In other parts of the body the hairs are generally shorter (0.028 to 0.050 mm.) On the anterior part of the cephalothoracic region, especially before the scutum, there is little hair. The hairs of the legs and mouth apparatus are not clubbed but simply pinnate and pointed, and on the whole similar to the pinnate hairs of *Trombidium fuliginosum* and our *Trombidia* B and C. The hairs are especially numerous on the first pair of legs and on the appendage of the palpus.

#### *The Prosopon of the Tsutsugamushi.*

Encouraged by the result of breeding the nymphs, we tried to find them in the endemic district, and succeeded after great difficulty. On July 13 we found 3 specimens in Horiguchi, 8 and 7 respectively on July 17 and 18 in Okiage, 7 on July 19 in Nakagawara, 1 on July 31 in Horiguchi, 15 on August 1 in Nakagawara, 1 on August 9, and 23 on August 14, making a total of 65 specimens. It requires great patience to search in the oppressive heat of summer, with the entire body carefully covered against the larval bite, for this tiny pale colored insect which can be distinguished from a grain of sand only by its movement. We often came home without results. One

day, when five persons took part in the hunt, only one specimen was found. The tsutsugamushi are usually found in the ground, and when brought to light by the turning of the soil, they try to hide again in the earth. Their color is sometimes grayish white without any yellowish red shade, but usually pale yellow or orange. In the latter case the hairs remain colorless. The body of the animals is always considerably larger than that of artificially bred nymphs as is shown in Table II.

TABLE II.

No.	Length.	Greatest width.		Color.
		Thorax.	Abdomen.	
	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	
1	1.166	0.564	0.720	Pale yellow.
2	0.738	0.415	0.476	Gray.
3	0.918	0.424	0.600	"
4	0.922	—	—	Orange-yellow.
5	About 1.000	—	—	Pale yellow.
6	" 1.000	—	—	Orange-yellow.
7	0.886	0.416	—	Gray.
8	1.152	0.612	—	"
9	0.810	0.450	0.486	Pale yellow.
10	1.080	0.540	0.700	" orange.
11	1.250	0.630	0.900	Orange.
12	1.260	0.620	0.810	"
13	0.950	0.584	0.680	Grayish white.
14	1.116	0.504	0.648	Pale orange.
15	0.990	0.396	0.414	Stained.
16	1.440	0.720	0.900	Orange.
17	1.260	0.594	0.756	"
18	1.170	0.504	0.648	"
19	1.440	0.840	1.080	Pale orange.
20	1.260	0.680	0.936	Orange.

When we first discovered these mites we were of the opinion that we had before us the free living nymphs of the tsutsugamushi, for we found with the exception of the difference in size and a deeper coloring in many specimens no essential difference in outward appearance from the artificially reared ones. But when we made a minute comparison, we found a difference in the number of sucking discs

of the genital opening. While the nymphs, as already described, always have two pairs, we counted in this case three pairs of discs. Banks (14) has already pointed out that in certain mites, especially in Hydrachnidæ which resemble Trombidia, the shape and number of the sucking discs are important characteristics in distinguishing the species. We found in Trombidia A and B always three pairs, in the nymph as well as in the prosopon, while Trombidium C has only two in the nymph and three in the mature animal.

It therefore seems that the animals caught by us were not nymphs but mature animals. We have hesitated to pronounce them as such until we succeeded in finding eggs in their body. In animals caught on July 31 and August 1 we found eggs in various stages, which could be distinctly seen by pressing out the contents of the stomach under a cover-glass. The mature eggs are round, and have a relatively thin shell and an almost colorless yolk; their diameter is about 0.162 mm. Besides these a few immature, mostly smaller eggs can be seen through the skin. But we did not find any prosopa rich in eggs. It is probable that they deposit their eggs only in small quantities; at any rate, it seems that they never deposit a single heap formed of hundreds of eggs, as is the case with other trombidia.

Measurements of the various parts of a prosopon 1.166 mm. long are given in Table III.

TABLE III.

	mm.
Width of thorax.....	0.504
"    "    abdomen.....	0.720
"    "    constricted part.....	0.468
Distance of the third pair of coxæ (epimeres).....	0.120
Length of mandibles.....	0.180
Width " ".....	0.068
Length " mandible hook.....	0.068
Total length of palpi with basal joint.....	0.344



TABLE III—*Continued.*

Palpi.		
		<i>mm.</i>
Basal joint.	{ Length.....	0.120
	{ Width.....	0.042
Joint 1.	{ Length.....	0.024
	{ Width.....	0.040
“ 2.	{ Length.....	0.084
	{ Width.....	0.044
“ 3.	{ Length.....	0.056
	{ Width.....	0.040
“ 4.	{ Length.....	0.060
	{ Width.....	0.024
Claw.....		0.020

Leg.					
		1	2	3	4
		<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
Coxa.	{ Length.....	0.120	0.080	0.112	0.112
	{ Width.....	0.056	0.048	0.090	0.090
Joint 1.	{ Length.....	0.064	0.048	0.044	0.052
	{ Width.....	0.048	0.044	0.040	0.044
“ 2.	{ Length.....	0.056	0.048	0.048	0.060
	{ Width.....	0.048	0.040	0.038	0.042
“ 3.	{ Length.....	0.088	0.048	0.044	0.060
	{ Width.....	0.060	0.040	0.038	0.044
“ 4.	{ Length.....	0.108	0.060	0.056	0.068
	{ Width.....	0.060	0.044	0.038	0.040
“ 5.	{ Length.....	0.112	0.064	0.068	0.088
	{ Width.....	0.064	0.044	0.038	0.040
“ 6.	{ Length.....	0.200	0.108	0.104	0.132
	{ Width.....	0.072	0.072	0.034	0.036
Claw.....		0.028	0.036	0.033	0.032
Total length of legs without epimeres.....		0.628	0.378	0.364	0.460

		<i>mm.</i>
Width of dorsal shield.....		0.072
Greatest sagittal diameter of dorsal shield.....		0.038
Length of tactile hair.....		0.120
Genital parts.	{ Length.....	0.113
	{ Width.....	0.080
Genital opening.	{ Length.....	0.060
	{ Width.....	0.036
Sucking discs.	{ Length.....	0.028
	{ Width.....	0.036
Anus.	{ Length.....	0.048
	{ Width.....	0.056
Distance between anus and genital opening.....		0.080
Length of dorsal hairs.....		0.056-0.068
“ “ hairs of legs.....		0.032-0.042



Morphologically the prosopa of the tsutsugamushi, differ greatly from other trombidia. Their chief characteristics are their small size, pale color of body, indistinct eyes and stigma. They are also characterized by the distinct constriction between the cephalothoracic region and the abdomen and the peculiarity of their hairs.

Sections have so far been made of six nymphs and twelve mature animals. Except for the genital gland both forms show in their inner morphology practically no differences. They have, compared with other species, a comparatively soft chitinous coat, so that for making serial sections a short immersion in alcohol or ether is sufficient. Both forms have a large brain and a lobed stomach. The intestine, esophagus, fat bodies, etc., are like those of the other trombidia. The large paired salivary glands possess several lobes and show three different kinds of cells according to their protoplasm or enclosed granule. One variety has fine granules which stain weakly with eosin, and the second coarse, distinctly eosinophilic granules, while the third consists of almost transparent cells filled with numerous vacuoles. Occasionally all three kinds of cells are found in the same lobe. At least two ducts are visible, which later unite. The Malpighian tubule consists of a pair of tubes. Both ends lie on the ventral side of the salivary gland and genital organ, the middle part being situated near the brain and the stomach.

The tracheal system is extraordinarily rudimentary, as are the eyes.

We were unable to find any tracheal capillaries, while in *Trombidia* A, B, C, and D numerous tracheas are easily seen in almost all parts of the body.

Regarding the genital gland, we found in the artificially bred nymphs only cell heaps directly behind the brain, between the genitalia and the stomach, which may be regarded as the undifferentiated germ cells. The animals caught in the open, on the contrary, show a well developed gland; *i.e.*, testicles or ovaries. In the female there is an oviduct on either side and a uterus provided with a muscular wall. The ovary contains ova in various stages of development. In three cases we found eggs already enclosed in the shell.

According to the external and internal morphological structure of the prosopa there is no possible doubt that we have before us the mature form of the tsutsugamushi.

We should mention that our nymph of the tsutsugamushi is closely related to that of *Leptus autumnalis*, artificially bred by Brandis (15), while the prosopon of the tsutsugamushi is different from *Trombidium holosericeum*; the latter, however, has, since Mégnin, often been considered the parent of *Leptus autumnalis*.<sup>1</sup>

*Cultivation of the Larva of the Tsutsugamushi.*

After we had discovered the prosopon of the tsutsugamushi in the open field (16), we tried to breed larvæ (tsutsugamushi) from the mature animal. To this end we distributed many prosopa into a number of small glass vessels and examined each vessel every 2nd day. On August 18 we found for the first time three small, yellowish red insects crawling about on the side of one of the vessels, which after being fixed in Berlese's fluid were found to be tsutsugamushi larvæ. The next day four larvæ were discovered in another vessel. No eggs were found in the soil of the two vessels, one of which on previous examination had been found free of trombidium eggs, while the other had been sterilized by heat. In the investigation of Trombidia A, B, C, and D we had found heaps consisting of hundreds of eggs. In one of the vessels we found the dead bodies of two prosopa. The hatched larvæ are undoubtedly the true tsutsugamushi; *i.e.*, they show no essential difference from tsutsugamushi larvæ taken from field mice and man; but they are distinctly smaller than those that have fed on the lymph of warm-blooded animals; they appear almost round in contrast to the more oval shape of the latter. Moreover, larvæ which have not yet sucked on mammals, have a broader mouth part and a more deeply colored body, which is orange-red in appearance. Otherwise the palpi, legs, epimeres, hairs, stigmata, eyes, and the appearance of the body are alike in both. Though we have not yet found eggs in the soil of the receptacle, it seems certain that the hatched larvæ are the offspring of the prosopa, because the soil had been thoroughly sterilized by heat before the latter were put in. There are two possibilities as to the way in which the larvæ develop; either the prosopa deposit so few eggs that they easily escape the attention of the observer, or no eggs are deposited, the larvæ leaving the body of the parent already formed

(viviparous). At any rate, by the artificial breeding of the larvæ the parent of the tsutsugamushi has been definitely established, and the tsutsugamushi is distinct in this as it is in the nymph and prosopon stages.

#### SUMMARY.

##### *Nomenclature.*

As mentioned above, the prosopon and the nymph of the tsutsugamushi have many characteristics which distinguish it from the other trombidia. These are the pale color and small size of the body, delicacy of the skin, the conspicuous constriction between the cephalothoracic region and the abdomen, rudimentary eyes and tracheal openings, absence of tracheal capillaries, etc. The fact that the thumb or the appendage of the palpus does not start from the side of the fourth joint of the latter but from its end, is another characteristic which does not occur in other trombidia. From the biological point of view two facts should be mentioned; *i.e.*, the adult female does not deposit eggs in heaps, and in contrast to those of other species, the larvæ hatch out all through the year and feed on mammals. From these characteristics running through all the stages of development, we believe that we are justified in claiming a new genus for the tsutsugamushi. We therefore propose to change the scientific name given to this mite by Brumpt in 1910, *Trombidium akamushi*, and to introduce instead, according to the suggestion of Drs. Goto and Watase, the name *Leptotrombidium akamushi*.

##### *Determination of Leptotrombidium akamushi, N. Sp.*

##### *Prosopon and Nymph.*

1. Pale color.
2. Delicacy of the skin.
3. Distinct constriction between the cephalothoracic region and the abdomen.
4. Rudimentary, sessile eyes.
5. Indistinctness of tracheal openings.
6. Absence of tracheal capillaries.
7. Crista abruptly widened at the end.
8. One palpal claw.

*Larva.*

1. One scutum.
2. Round spiracles.
3. Legs, six joints, not counting the coxa.
4. Hairs on the coxa: one each on Joints 1, 2, and 3.
5. Hairs on the scutum, including two tactile hairs, seven; one is in the median line.

*Specific Determination of Leptotrombidium akamushi, N. Sp.*  
(*Trombidium akamushi* Brumpt, 1910).

*Prosopon.*

1. Small size.
2. Three pairs of sucking discs on the external genitalia.
3. Hairs of the body feathered, club-shaped at the end, and colorless.
4. The appendage of the palpus tapers distinctly towards the end.

*Nymph.*

1. The size of the body is smaller than that of the prosopon.
2. Two pairs of sucking discs on the external genitalia.

There are no other distinct differences in the external morphology between the prosopon and the nymph.

*Larva.*

1. The color of the body is orange-red, but becomes paler after sucking on mammals.
2. The hairs of the body average 110 to 120 in number.
3. One pair of double eyes.
4. The sucking tube is visible when feeding on mammals (Hayashi).
5. The salivary gland is relatively large (Kawamura and Komagata).
6. The number and arrangement of the hairs on the legs and mouth parts correspond to the description given by Hirst.
7. The larvæ are found all through the year.
8. The larva is the carrier of tsutsugamushi disease in man.

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## EXPLANATION OF PLATES.

## PLATE 24.

- FIG. 1. Tsutsugamushi larva artificially bred.  $\times 143$ .  
FIG. 2. Tsutsugamushi larva from man, fully fed.  $\times 135$ .  
FIG. 3. Tsutsugamushi nymphophane on the 2nd day of breeding.  $\times 79$ .  
FIG. 4. Tsutsugamushi nymphophane on the 8th day of breeding.  $\times 79$ .  
FIG. 5. Tsutsugamushi nymph artificially bred from larva, seen from the ventral side.  $\times 79$ .

## PLATE 25.

FIG. 6. Tsutsugamushi nymphophane on the 7th day of breeding. The original larval skin is pierced by a spur-like structure of the nymphophane (left side upwards).  $\times 220$ .

FIG. 7. Tsutsugamushi nymph artificially bred from larva.  $\times 200$ .

## PLATE 26.

FIG. 8. Tsutsugamushi prosopon (captured specimen).  $\times 81$ .

FIG. 9. Tsutsugamushi. Sagittal section of a male prosopon.  $\times 97$ .

## PLATE 27.

FIG. 10. Tsutsugamushi. Sagittal section of a female prosopon.  $\times 100$ .

FIG. 11. Field work in Okiage, one of the endemic districts in Yamagata ken, July 18, 1916.

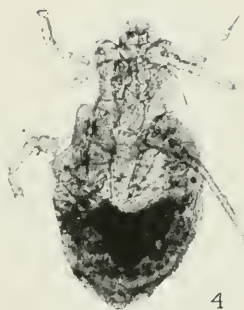




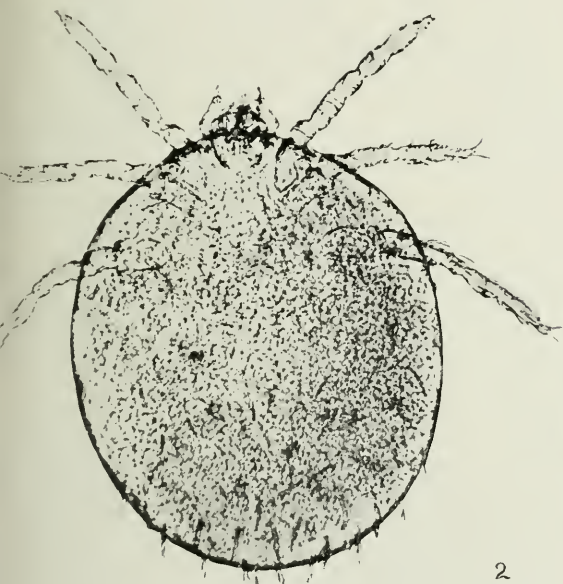
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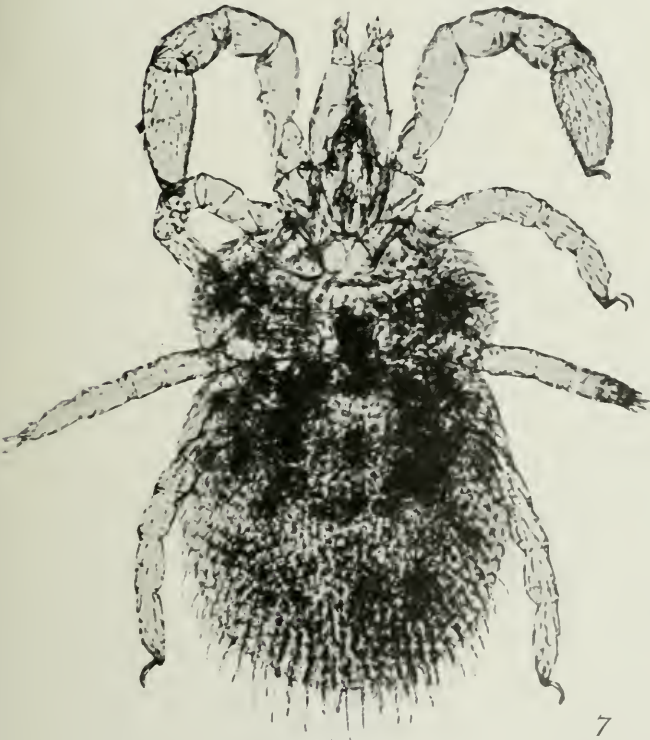
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(Nagayo, Miyagawa, Mitamura, and Imamura: The Tsutsugamushi.)





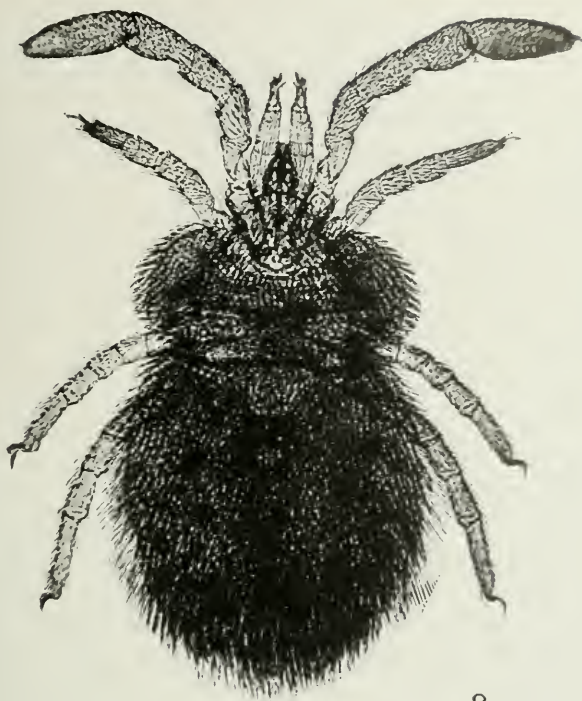
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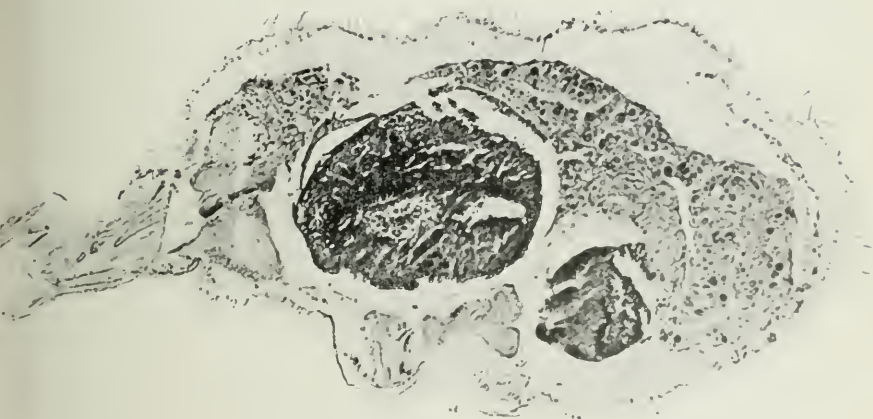
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(Nagayo, Miyagawa, Mitamura, and Imamura: The Tsutsugamushi.)





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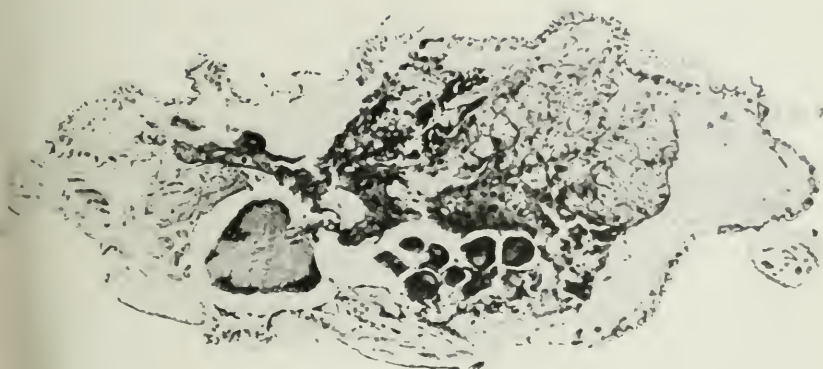


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(Nagayo, Miyagawa, Mitamura, and Imamura: The Tsutsugamushi.)







10



11

(Nagayo, Miyagawa, Mitamura, and Imamura: The Tsutsugamushi.)



# IS TROMBIDIUM HOLOSERICEUM THE PARENT OF LEPTUS AUTUMNALIS?

BY MATARO NAGAYO, M.D., YONEJI MIYAGAWA, M.D., TOKUSHIRO  
MITAMURA, M.D., AND ARAO IMAMURA, M.D.

(From the Imperial Institute for Infectious Diseases, Tokyo, Japan.)

PLATE 28.

(Received for publication, October 11, 1916.)

The question whether *Trombidium holosericeum* Linné, is the parent of *Leptus autumnalis* Latreille, (*Leptus autumnalis* Shaw) does not affect our work on tsutsugamushi directly. But indirectly, in the investigation of the life cycle of the tsutsugamushi, the carrier of tsutsugamushi disease, the question is of importance, because the tsutsugamushi, or akamushi, is almost identical with the European *Leptus autumnalis*. From our observations and from a study of the literature we cannot accept the view that *Trombidium holosericeum* is the parent of *Leptus autumnalis*.

Mégnin (1) first stated that *Trombidium holosericeum* was the parent of *Leptus autumnalis*, the latter having been studied by Latreille, de Geer, Gervais, and others before him. He observed that *Trombidium holosericeum* first appeared on the ground in April and was laden with numerous eggs from the end of May till June. He further succeeded in raising larvæ from these eggs, which according to him were *Leptus autumnalis* or the redbug. Mégnin does not state whether his morphological description of the larvæ is made from the larvæ artificially bred from *holosericeum* eggs. But it seems to us probable that Mégnin described the larva only from *Leptus autumnalis* which he had taken from warm-blooded animals, such as the rabbit, dog, etc., but not the bred *holosericeum* larvæ. In any case a close morphological comparison seems necessary for the identification of the larvæ, for it seems to us possible that Mégnin's artificially bred larva may have belonged to another species of trombidium.

When we first saw the larva of *Trombidium* B<sup>1</sup> bred from eggs we

<sup>1</sup> Nagayo, M., Miyagawa, Y., Mitamura, T., and Imamura, A., On the Nymph and Prosopon of the Tsutsugamushi, *Leptotrombidium akamushi*, N. Sp. (*Trombidium akamushi* Brumpt), Carrier of the Tsutsugamushi Disease, *J. Exp. Med.*, 1917, xxv, 255.

had no doubt that it was the *tsutsugamushi*, and that thus the parent of the latter was established. Later, however, we perceived inconspicuous differences, which nevertheless were essential differentiating characteristics between the two larvæ. The larva of *Trombidium* B is distinguished from the *tsutsugamushi* by the much smaller number of hairs, the localization and arrangement of the latter, the shape of the stigmata and of the mouth apparatus, as well as the length and the number of joints of the legs, etc. Mégnin does not give a detailed comparison but his statement that the *holosericeum* larva is identical with *Leptus autumnalis* has, nevertheless, been frequently cited. As far as we know, this statement has not been confirmed by reinvestigation; that is, by breeding *Leptus autumnalis* from the eggs of *Trombidium holosericeum*. Even Brandis (2), who found *Leptus autumnalis* in great numbers at Nietleben near Halle a. S. and for the first time was able to breed the nymph of the latter, does not mention the mature animal or eggs. Perhaps he did not succeed in finding *Trombidium holosericeum* or its eggs in the place where he had found many larvæ. His successful breeding of the nymph, which according to his sketches and description, closely resembles our *tsutsugamushi* nymph, is of great importance but this is not proof of Mégnin's statement. We have found since April, 1916, fifteen specimens of a trombidium species, which we have provisionally named *Trombidium* D, on the ground in various parts of Yamagata ken. Morphologically and biologically they are identical with *Trombidium holosericeum*. Their color is carmine red and they show the typical silky luster. They are 2.34 to 3.50 mm. long and 1.80 to 2.70 mm. broad. This trombidium is somewhat square, with a sagittal incision in the hind end of the body and with several grooves in the back. The hairs are long and pinnate, with clubbed ends; in the joints of the legs they are a little longer. The legs have two claws, without clinging hairs. The eye has a long peduncle. Since May these animals have laid several heaps of eggs in the vessels. The eggs measure from 0.15 to 0.18 mm., usually 0.176 mm. They are almost spherical, pale yellow, and shining, and gradually assume an orange-yellow color. Several larvæ appeared at the end of 3 weeks in the incubator at 22°C. These larvæ have a mouth apparatus somewhat resembling that of *Leptus autumnalis* and of *tsutsu-*

gamushi (Figs. 1 and 2), but there are also great differences, as the following table shows.

Larva of Trombidium D ( <i>Trombidium holosericeum</i> ).		Tsutsugamushi.	<i>Leptus autumnalis</i> .
Hairs of the body.	Not numerous (about 48); 2 pairs of long hairs at the hind end.	Numerous (about 110 to 120); all about equally long.	Numerous; all about equally long.
Dorsal shield.	Two.	One.	One.
Stigma.	Oval.	Round.	Round.

It is not necessary to go into more detail, as the points mentioned show that the larvæ of *Trombidium* D, *i.e.*, *Trombidium holosericeum*, are different from both *Leptus autumnalis* and tsutsugamushi. In Oudemans's classification, the larva of *Trombidium* D belongs to *Allotrombidium*, and the two other larval forms to *Trombidium*, according to the number of dorsal shields.

Mégnin further observed that *holosericeum* larvæ immediately after hatching parasitized on warm-blooded animals as well as on insects. He states: The larva, which is almost spherical when it emerges, and much less active than that of *Trombidium fuliginosum*, and with shorter claws, is the same as the common redbug, *Leptus autumnalis*, which as soon as it is hatched attaches itself to some animal or insect. If this is the case, Mégnin's *holosericeum* larva cannot be regarded as a genuine *Leptus autumnalis*, because Brandis (2), as well as others, has made different observations. Brandis says that if the mites are shaken by a human being or a warm-blooded animal they immediately attach themselves to it.

There is perhaps no mammal, which comes within their reach, unmolested by them; they have been found on hares, rabbits, various kinds of mice, badgers, hedgehogs, molebat, shrew, dogs, and cats. On birds, reptiles, insects, and spiders, I could, however, not effect any infestation, though on insects and spiders near relations of *Leptus autumnalis* parasitize.

Our tsutsugamushi attacks field mice, rabbits, guinea pigs, monkeys, and other mammals, but not insects. They are biologically and morphologically closely related to *Leptus autumnalis*, though recently some minor differences in the form of the dorsal shield, in

the hairs of the palpi, etc., have been emphasized by Hirst (3). The behavior of the two nymphs is also similar, as mentioned above. For these reasons we cannot accept the statement that *Trombidium holosericeum* is the parent of *Leptus autumnalis*. The true parent of *Leptus autumnalis* must be another species, which, as far as we can find in the literature, has not been described as yet. We believe that the prosopon of *Leptus autumnalis* must be similar to that of *tsutsugamushi*.

A detailed report on the nymph and the prosopon of the *tsutsugamushi* was given in the preceding paper,<sup>1</sup> to which we may refer the reader.

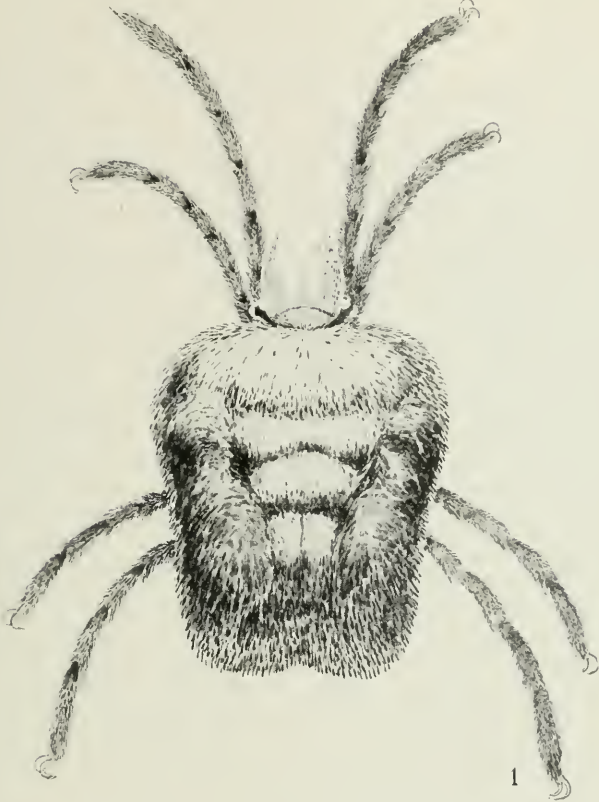
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2. Brandis, Ueber *Leptus autumnalis*, Festschrift Prov.-Irren-Anstalt zu Nietleben bei Halle a. S., 1897, 417.
3. Hirst, S., On the "Harvest Bug" (*Microtrombidium autumnalis* Shaw), *J. Econ. Biol.*, 1915, x, 73.
4. Hirst, On the *Tsutsugamushi* (*Microtrombidium akamushi* Brumpt), *J. Econ. Biol.*, 1915, x, 79.

#### EXPLANATION OF PLATE 28.

- FIG. 1. *Trombidium D* (*Trombidium holosericeum*), adult.  $\times 20$ .  
FIG. 2. *Trombidium D*, larva hatched from egg.  $\times 150$ .





1



2

(Nagayo, Miyagawa, Mitamura, and Imamura: *Trombidium holosericeum*.)



## A CONTRIBUTION TO THE STUDY OF PANCREAS INTOXICATION.\*

By E. W. GOODPASTURE, M.D.

(From the Peter Bent Brigham Hospital and the Harvard Medical School, Boston.)

(Received for publication, October 17, 1916.)

Two main views are held regarding the nature of the toxic agent of the autolyzing pancreas. One identifies it with active trypsin,<sup>1</sup> the other with split products of protein resulting from trypsin digestion.

Lattes<sup>2</sup> has recently shown that trypsinogen may be activated by some substance present in the dead pancreas cells, so that trypsin digestion may take place fairly rapidly in the autolyzing gland. This fact might of course be cited in favor of either of the two views, though Lattes himself is inclined to consider protein split products the active toxic agent.

Petersen, Jobling, and Eggstein<sup>3</sup> have shown that in acute intoxication from experimental hemorrhagic pancreatitis, the non-coagulable nitrogen of the blood is increased, indicating the absorption of material of a protein nature. Whipple<sup>4</sup> has isolated from the pancreas in which an acute experimental hemorrhagic necrosis had been produced, a toxic proteose which he considers an active agent in this disease.

The following study was undertaken<sup>5</sup> with the idea of purifying as far as possible the toxic part of the autolyzing gland. It was found that fresh pancreas extract in salt solution is toxic, and when injected intravenously produces symptoms of poisoning analogous

\*Aided by a grant from The Rockefeller Institute for Medical Research.

<sup>1</sup> von Bergmann, G., and Guleke, N., *Zur Theorie der Pankreasvergiftung*, *Münch. med. Woch.*, 1910, lvii, 1673.

<sup>2</sup> Lattes, L., *Über Pankreasvergiftung*, *Arch. path. Anat. u. Physiol.*, 1913, ccii, 1.

<sup>3</sup> Petersen, W., Jobling, J. W., and Eggstein, A. A., *J. Exp. Med.*, 1916, xliii, 491.

<sup>4</sup> Whipple, G. H., *J. Am. Med. Assn.*, 1916, lxvi, 2031.

<sup>5</sup> This investigation was begun with Dr. G. H. Whipple, at the Hunterian Laboratory of the Johns Hopkins Medical School.

to those resulting from duodenal loop fluid, and other toxic materials of a protein nature. The symptoms, however, develop relatively slowly, and reach their maximum about 2 or 3 hours after injection.

*Method of Preparing Fresh Extract.*

The pancreas is removed from a dog with aseptic precautions. It is cut finely and ground in a mortar with clean sand. 50 cc. of normal salt solution are added and mixed thoroughly with the macerated gland by grinding. The material is then filtered through gauze and cotton. The filtrate injected slowly intravenously into a dog causes a rapid fall in blood pressure with a more gradual but prompt return to normal or above. The clotting time of the blood is delayed and there is a profound fall in leukocytes. In from 1 to 3 hours after the injection, symptoms of extreme intoxication appear. These are persistent vomiting of bile-stained mucus; profuse diarrhea which becomes bloody; often bleeding from the gums; fall in blood pressure and in body temperature; slow, deep respiration; weak, rapid heart beat; coma and death if the dose is sufficient. If the dose is not lethal, the animal rapidly recovers, the temperature and leukocytes rise to above normal, vomiting and diarrhea subside, so that within 12 hours after the injection, the animal may appear normal, after a very severe intoxication.

Autopsy upon a fatally poisoned animal reveals extreme splanchnic congestion, especially apparent in the upper portion of the small intestine, the mucosa of which is deep purple in color from congestion and hemorrhage. Numerous focal necroses or hemorrhages are present in the liver. The gall bladder is edematous. The stomach and intestine are filled with bile-stained juices and mucus. By this method about one fatal dose may be obtained from a gland, for a dog of the same weight as the one from which the pancreas was obtained.

Treated rapidly in this manner, there can be little if any active trypsin present in the injected material. It seemed probable, therefore, that trypsin was not the active toxic agent, but some other substance present in the normal gland. Experiments with this idea in view revealed the fact that the  $\beta$ -nucleoprotein fraction of the gland is toxic and produces reactions identical with those following injection of the salt solution extract.

*Preparation of  $\beta$ -Nucleoprotein from Pancreas.*<sup>6</sup>

Fresh pancreas is chopped finely and ground in a mortar with sand, thoroughly stirred with twice its weight of distilled water, and heated to the boiling point for 10 minutes. The interstitial fluid is poured off and filtered, while hot, through ordinary filter paper, and the residue is again heated to boiling with one-half the original volume of water. This is filtered hot and the residue squeezed in gauze. The two filtrates are united. The fluid is opalescent. It is again filtered, and acidified until it contains 1 per cent acetic acid. A creamy white flocculent precipitate forms. It is cooled in running water and the precipitate completely thrown down by adding an equal volume of 95 per cent alcohol. It is placed in the ice box for 6 to 12 hours, and the supernatant fluid is then filtered or centrifuged off. The precipitate is redissolved in distilled water, made slightly alkaline with dilute sodium carbonate, and reprecipitated by rendering it slightly acid with dilute acetic acid. This process is repeated several times to get rid of soluble impurities. The precipitate is then washed with alcohol and ether, and dried in a desiccator. Of this material 0.1 gm. per kilo produces a profound intoxication and sometimes death in young animals. It is dissolved in 10 or 20 cc. of very dilute sodium carbonate and injected intravenously.

The fact that the  $\beta$ -nucleoprotein fraction of the dog's pancreas is toxic proves the presence of a poisonous material in the fresh gland which cannot be active trypsin since the substance is thermostable. The  $\beta$ -nucleoprotein, however, contains, besides its protein constituents, guanylic acid.

A pure preparation of guanylic acid<sup>7</sup> from yeast nucleic acid was found to be non-toxic for dogs in doses of 0.1 gm. per kilo when injected intravenously. Although the guanylic acid prepared by Bang<sup>8</sup> from beef pancreas was toxic for dogs, he was no doubt working with a relatively impure product.

<sup>6</sup> Steudel, H., and Brigl, P., Über die Guanylsäure aus der Pankreasdrüse, II, *Z. physiol. Chem.*, 1910, lxxviii, 40.

<sup>7</sup> This material was obtained through the kindness of Dr. W. Jones.

<sup>8</sup> Bang, I., Chemische und physiologische Studien über die Guanylsäure, *Z. physiol. Chem.*, 1901, xxxii, 201.

It seemed from these experiments that the toxic material of the normal pancreas is the protein constituent of  $\beta$ -nucleoprotein, or is in some way associated with it.

When fresh dog pancreas was autolyzed for 12 to 24 hours with chloroform and toluene at 37°C., the autolysate was found to be toxic, though less so than the fresh gland, and after autolyzing for longer periods the toxicity disappeared. An attempt was then made to recover the toxic fraction from the autolyzed gland, and by this method a product has been obtained which is more toxic than the  $\beta$ -nucleoprotein.

*Preparation of Toxic Material from the Autolyzed Pancreas.*

Ground fresh pancreas is mixed with twice its weight of distilled water made slightly alkaline to litmus with sodium carbonate. About 1 cc. of chloroform is added and the mixture covered with toluene. It is placed in the thermostat and digested at 37°C. for 12 to 24 hours, being shaken at intervals. It is then made acid to litmus with dilute acetic acid, boiled for 10 minutes, and filtered hot through an ordinary filter paper. The clear yellow filtrate is made acid up to 2 per cent acetic, cooled in running water or in the ice box, and left to stand for several hours. A flocculent precipitate forms. This is centrifuged and the supernatant, slightly cloudy fluid is decanted. The precipitate is redissolved in dilute alkali and reprecipitated by acidifying with a few drops of 1 per cent acetic acid. This is repeated until the decanted fluid gives no cloud with a drop of 1 per cent hydrochloric acid. It is then washed with alcohol and ether and dried in a desiccator. This material is brownish, rather tough, easily soluble in warm dilute alkali, and has a tendency to gel on cooling in very dilute alkaline solution. It gives a strong, light purple biuret reaction. In doses of from 0.05 to 0.1 gm. per kilo of body weight intravenously or intraperitoneally in dogs, it produces severe intoxication which is usually lethal in young animals. The symptoms are those described as following an injection of fresh extract of pancreas.

The method of recovery of this toxic protein fraction from the autolyzed gland discloses a difference in the enzymatic activity in the auto-



lyzing pancreas of the dog, as contrasted with that of beef or pig. The product was recovered in such small quantity and there was such difficulty in obtaining dog material that attempts were made to procure a similar substance from beef and pig pancreas. While the  $\beta$ -nucleoprotein from these glands was found to be toxic, no poisonous material could be recovered after autolysis by the method used with dog pancreas.

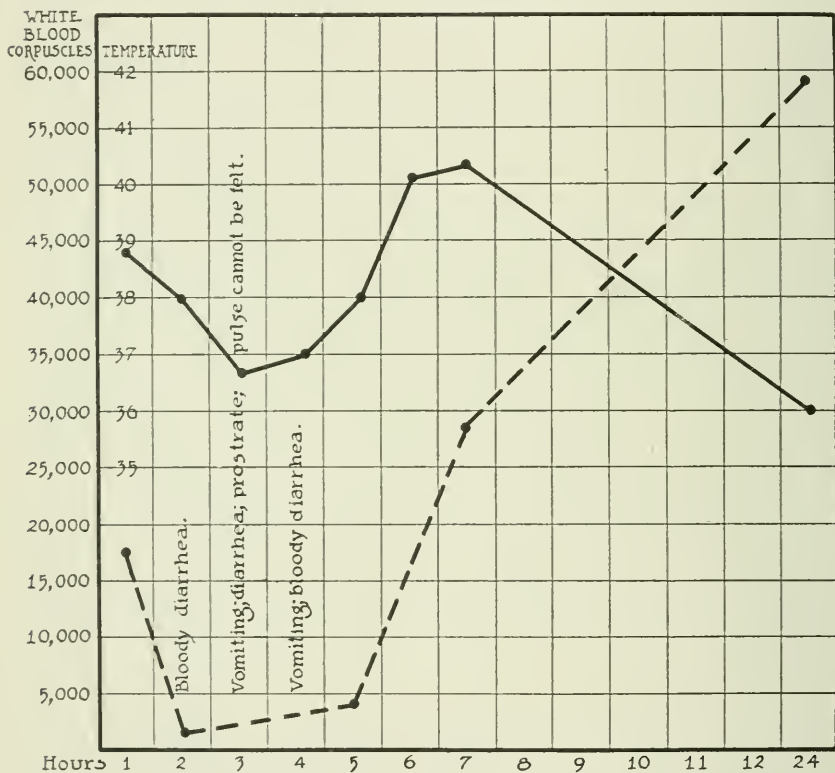
After the toxic fraction of dog pancreas is precipitated from the autolytic filtrate by acetic acid and filtered off, an abundant, white, flocculent precipitate can be thrown down from this second filtrate by the addition of a few drops of 1 per cent hydrochloric acid and an equal volume of 95 per cent alcohol. This material when purified by redissolution and reprecipitation with dilute hydrochloric acid, washed in alcohol and ether, and dried, is a granular sandy substance, which becomes a white powder when ground in a mortar. It is biuret-free, and is non-toxic for dogs in doses of 0.1 gm. per kilo. It contains an abundance of phosphorus and guanine. It gives a deep pentose color reaction and does not seem to contain adenine. In all probability it is largely composed of guanylic acid.

It is well known that guanylic acid disappears rapidly from the autolyzing pancreas of beef or pig, and our experiments have shown that the toxic protein material also cannot be recovered from these glands, whereas a poisonous protein fraction and especially guanylic acid can be obtained in relatively large amounts from autolyzing dog pancreas.

Since  $\beta$ -nucleoprotein is toxic and guanylic acid is not, we have assumed that the toxic material is identical or closely associated with the protein portion. Both the protein portion and guanylic acid are rapidly destroyed in beef and pig pancreas, while neither is attacked or very slowly attacked during autolysis of dog pancreas. But it is possible after autolysis, to separate the two and recover the toxic substance in greater concentration.

Petersen, Jobling, and Eggstein's<sup>3</sup> observations upon the serum changes during intoxication from acute hemorrhagic pancreatitis suggest that poisoning results from absorption of protein material, and it seems more probable that the protein itself which we have isolated is a toxic agent than that some other material is associated with it.

The separation by Whipple<sup>4</sup> of a toxic proteose from the glands in hemorrhagic pancreatitis indicates that the protein substance I have isolated is probably not the sole active agent in the production of toxic symptoms in this disease. Perhaps many different injurious



TEXT-FIG. 1. Temperature and leukocyte chart of a dog following the intravenous injection of 0.05 gm. of toxic protein of the pancreas per kilo of body weight. The solid line represents the temperature, the broken line the number of leukocytes.

substances may be absorbed from such a complex material as an autolyzing pancreas.

Text-fig. 1 summarizes an experiment in which the concentrated toxic protein was injected intravenously. Because of the small quantity of material available, pups weighing from 6 to 8 pounds

were used. The purified dry substance was dissolved in 10 cc. of very dilute sodium carbonate and injected rapidly into the jugular vein. 50 mg. per kilo of body weight were used. The animal recovered.

#### CONCLUSIONS.

1. A toxic constituent is present in the fresh pancreas of dogs before trypsinogen can have been activated.

2. The toxic fraction is present in the  $\beta$ -nucleoprotein, and therefore is thermostable.

3. It is gradually destroyed by autolysis but can be obtained in concentrated form from a gland which has autolyzed 24 hours.

4. The toxic material is probably protein in nature.

5. Intravenous or intraperitoneal injection of from 0.05 to 0.1 gm. per kilo of body weight is usually fatal and elicits in dogs symptoms comparable to those of spontaneous hemorrhagic necrosis of the pancreas.

6. Dog pancreas differs from that of beef or pig in that guanlyic acid and toxic protein combined with it are slowly broken down by autolysis.

7. After autolysis of dog pancreas the toxic protein and guanlyic acid may be separated and obtained in relatively pure form.



# THE CYCLIC CHANGES IN THE MAMMARY GLAND UNDER NORMAL AND PATHOLOGICAL CONDITIONS.

## I. THE CHANGES IN THE NON-PREGNANT GUINEA PIG.

BY LEO LOEB, M.D., AND CORA HESSELBERG, M.D.

*(From the Department of Comparative Pathology of Washington University  
Medical School, St. Louis.)*

(Received for publication, November 10, 1916.)

### INTRODUCTION.

While the literature dealing with the growth and function of the mammary gland is extensive, the mechanism determining the growth processes as well as secretion is incompletely known. Growth and function of the mammary gland are so closely interrelated with the cyclic changes of the uterus and ovaries that an understanding of the latter is a prerequisite for the former.

During the last 10 years one of the writers has brought to a conclusion the analysis of the mechanism of the mammalian, uterine, and ovarian cycle in its principal outlines. On the basis of the previous studies it is now possible to attempt a further analysis of processes of growth and secretion in the mammary gland. While the results in this and the succeeding paper clear up certain phases of the problem, it will be necessary in further studies to complete the analysis.<sup>1</sup>

The conclusions embodied in this and the succeeding paper are based on the examination of the mammary glands in guinea pigs. In almost all the animals examined the period of heat had been

<sup>1</sup> During the summer of 1915 a number of the mammary glands were prepared for microscopic examination in the Pathological Laboratory of the St. Louis University Medical School. The facilities of this laboratory were generously placed at our disposal by the authorities of the School and by the Director of the laboratory, Dr. Ralph L. Thompson. We take this opportunity to express our appreciation of the assistance accorded us.

observed, and the period of the sexual cycle of the animal was therefore known. It was confirmed by microscopic examination of the ovaries and uterus in the majority of cases; the former were invariably cut in serial sections and thus a comparison between the condition of the ovaries and uterus on the one hand, and of the mammary gland on the other, was made possible. In addition, the majority of the animals were subjected to certain experimental procedures, under ether anesthesia, at a known period of the sexual cycle, in order to determine the significance of the ovaries as a whole, of the corpora lutea, of the uterus, of the deciduomata, with and without pregnancy, and of lutein injection for the condition of the mammary gland.

In order to avoid the necessity of describing in detail in each case the state of the mammary gland as revealed on microscopic examination, we characterized the mammary gland as proliferating, as resting, or as intermediate. We disregarded almost entirely the amount of gland tissue which we found at the time of examination. The latter depended not so much on the stage of the cycle in the mammary gland and on the experimental interferences as apparently on more or less accidental factors, particularly on the time which had elapsed since the last pregnancy preceding the time of examination. We shall describe briefly the different types of mammary glands.

*Proliferating Mammary Gland.*—The acini and large ducts are of variable size, sometimes very large, and even cystically dilated. The epithelium is high cuboidal or almost cylindrical; in some instances it is only medium cuboidal. The epithelial nuclei are vesicular. There are mitoses present in the acini, sometimes in large numbers, in other glands they are less frequent; in some cases they are only rarely found. The amount of colloid-like material within the acini or ducts varies in different glands. There may be papilla-like protrusions into the large ducts. The connective tissue between the acini is scant, more or less cellular, with large, sometimes with smaller vesicular nuclei. Lymphocytes and polynuclear leukocytes are usually found in the stroma. There is usually much fluid present in the stroma. In some glands mitoses are found in the fibroblasts of the stroma; in others they are absent. The amount and character of the stroma are not identical in all glands;



there may occasionally be some stronger development of fibrous tissue, especially in certain parts of the gland; the gland may be proliferating only in parts.

*Resting Mammary Gland.*—We usually find small acini, occasionally cystically dilated acini, lined by low cuboidal or flat epithelium, with dark staining, condensed or small vesicular nuclei. The gland ducts have likewise low epithelium. Desquamated epithelial cells may be present in the lumen of the acini. Colloid-like material may be present or absent. Mitotic proliferation is entirely lacking. The connective tissue between the acini or around the ducts is usually densely fibrous and often present in a relatively large amount. The nuclei in the stroma are small and compressed. Mitoses are absent. Polynuclear leukocytes and lymphocytes are usually not seen.

*Intermediate Mammary Gland.*—While the types of proliferative and resting mammary gland are fairly sharply defined, this is not so in the same degree in the third type, the intermediate mammary gland. Here mitoses are always absent, but the structure of the gland and stroma does not approach the inactivity of the resting gland, but approaches more or less that of the proliferating gland. The gland epithelium is higher than in the resting gland, and the stroma, in parts or throughout, is more cellular than in the resting gland; the nuclei are more vesicular, and the quantity of dense hyaline connective tissue, throughout the gland or at certain places, is much diminished. Lymphocytes and leukocytes are often present, but the characteristic feature of the proliferating gland, the mitoses, are absent. The intermediate gland may otherwise bear a greater resemblance to the proliferating or to the resting gland and we may therefore make further subdivisions, designating some glands as intermediate to resting, others as better than intermediate.

*Normal Cycle of the Mammary Gland in the Non-Pregnant Animal.*  
*Period from Time of Heat to 5 Days after Ovulation.*

In the guinea pig the stage of heat can be recognized by examination of the ovaries and uterus. A detailed description of these changes has been previously given by one of us.<sup>2</sup> In the observations

<sup>2</sup> Loeb, L., *J. Morphol.*, 1911, xxii, 37; *Virchows Arch. path. Anat.*, 1911, ccvi, 278; *Biol. Bull.*, 1914, xxvii, 1.

reported in this communication the diagnosis of the period of heat is based on the state of these organs as shown by microscopical examination. In some cases the objective signs of heat were not yet completely present, but even in these cases, knowing that the interval since the preceding ovulation corresponds to a full period of the sexual cycle, and finding those changes in the uterus and ovaries which approach the characteristics of heat, we were in a position to diagnose an impending heat.

*Mammary Gland near the Stage of Heat or during Heat.*—We examined the mammary gland in seven guinea pigs which were either in heat or near heat. In all of them we found proliferating glands; in no case was the gland in a resting or intermediate condition. In the guinea pig the period of heat is usually followed by ovulation. The latter generally occurs 6 to 12 hours after copulation, but in the guinea pig a preceding copulation is not, as it is in some animals, a prerequisite for ovulation. The time immediately preceding or following ovulation can be recognized by a microscopic examination of the ovaries, which shows greatly enlarged mature follicles either near the stage of rupture or directly following rupture.

*Mammary Gland near Ovulation or within 24 Hours after Ovulation.*—We examined the mammary glands of eight guinea pigs which were either at the point of ovulation or in which ovulation had taken place within the past 24 hours. In five of these animals the glands were proliferating. In three of the latter animals incisions had previously been made into the uterus and deciduomata had developed, which at the time of examination had become necrotic. Ovulation, however, had not been delayed by the presence of necrotic deciduomata. In two other cases the gland showed an intermediate character, and in one case we found a gland in the resting state. In the latter instance, incisions had been made in the uterus, and deciduomata had developed and caused a delay in ovulation. It was  $26\frac{1}{2}$  days after the previous ovulation before a new ovulation took place; the deciduomata were found necrotic soon after ovulation. In this case of delayed ovulation a resting gland was found in spite of the period of the sexual cycle at which the examination had taken place. Here apparently the delay in ovulation caused by the pres-

ence of deciduomata was responsible for the lack of progressive changes in the mammary gland.

*Mammary Gland from 24 to 72 Hours after Ovulation.*—We examined sixteen guinea pigs in this period of the sexual cycle. In seven we found a proliferating gland. In one of these a necrotic deciduoma was present, but ovulation had taken place at the proper time. In seven animals the glands were intermediate; in two of these we found necrotic deciduomata but there had not been any delay in ovulation. In two animals the glands were resting, or slightly better. In both of these necrotic deciduomata were present. In one of the animals ovulation had been delayed, and in the second it had taken place at the proper time. We found, therefore, a proliferating gland in not quite one-half of the animals, but a resting gland only in a small minority.

*Mammary Gland from 72 Hours to the End of the 5th Day after Ovulation.*—At this stage of the sexual cycle we find six proliferating glands. No incisions in the uterus had been made and no deciduomata had, therefore, developed. In five animals the glands were intermediate; ovulation had taken place at the proper time in three, and the remaining two died a few days after ovulation. In no case had incisions been made into the uterus. In five animals the glands were somewhat better than resting, but not quite intermediate. At this period a little more than one-third of the glands were proliferating and almost one-third were slightly better than resting.

If we summarize the results, we find that during a period of the sexual cycle including the time preceding heat until 5 days after ovulation, there is a gradual decrease in the activity of the mammary glands. At the time of heat all the glands proliferate, and from 3 to 5 days after ovulation, little more than one-third are proliferating and almost one-third are near the resting state. There is a maximum at the period of heat and from then on there is a gradual decline to the end of this period. In some instances in which a delay of ovulation had been experimentally produced, the mammary gland seemed to show less activity during this period than in the animals in which ovulation took place at the usual time. Our observations, however, merely suggest the latter conclusion.

*Period from 5 to 15 Days after Ovulation.*

*Mammary Gland from the Beginning of the 6th to the End of the 12th Day after Ovulation.*—Twenty-nine animals in this period of the sexual cycle were examined. There was in addition one guinea pig which presumably belonged to this stage. Of these, fifteen or sixteen animals showed an intermediate, and fourteen an approximately resting condition of the mammary gland; the majority of the latter were slightly better than resting. In no case was a necrotic deciduoma present in the uterus. A proliferating gland was not found during this period. Almost half the glands were, therefore, in a resting, and the rest in an intermediate condition.

*Mammary Gland from the Beginning of the 13th to the End of the 15th Day after Ovulation.*—Four animals were examined in this stage of the sexual cycle. One showed a proliferating mammary gland. In this case a foreign body had been introduced into the uterus  $2\frac{1}{2}$  days after heat; 6 days after heat the foreign body was removed and incisions were made into the uterus. Three good corpora lutea were found in the ovaries. In three other animals the mammary glands were resting or slightly better than resting. In the first of these a good corpus luteum and mature follicles were present. In the second we found good corpora lutea as well as a small deciduoma and mature follicles. In the third we found partly preserved, partly necrotic deciduomatous tissue, and lutein injections had been made.

In this stage of the sexual cycle the mammary gland is, therefore, on the whole inactive. We may furthermore conclude that at this stage of the cycle neither mature follicles nor well preserved corpora lutea nor the presence of a small deciduoma are usually able to stimulate the mammary gland to activity, although in one case where good corpora lutea were present, proliferation had taken place.

If we summarize the findings during this whole period, we may conclude that the time from the 6th to the 15th day after ovulation represents the lowest level in the activity curve of the mammary gland, although good corpora lutea are usually present and mature follicles may appear in the second half of this period.

*Period from 16 to 27 Days after Ovulation.*

The normal sexual cycle in the guinea pig varies between 15 and 19 days. This series includes, therefore, not only guinea pigs in the last stage of the sexual cycle, but also animals in which normal ovulation had been delayed.

*Mammary Gland from the Beginning of the 16th to the End of the 19th Day.*—In this period we find five proliferating glands. In three of these cases the uterus had been extirpated and the corpora lutea were well preserved. In the two other cases vacuolar corpora lutea were present. Mature follicles were noted in two of the five cases. In four cases the mammary glands were resting. In one of these animals the ovaries were hypotypical; in three, good deciduomata were present. In two of the latter the ovaries had not been examined; and in the third the corpora lutea showed beginning vacuolization and mature follicles were present. In two animals the glands were intermediate. In one of these the structure of the gland approached that of a proliferating gland, but mitoses were not definitely observed. The condition of the uterus and ovaries indicated that this animal was near the period of heat. In the second case vacuolar corpora lutea and reactive follicles were present. But there was not yet any other indication of approaching heat. In this case the mammary gland at one place approached the proliferating stage. We find then that at this stage of the sexual cycle approximately one-half the animals had a proliferating gland. Proliferation occurs at this period in cases in which, while a new ovulation is not approaching, the corpora lutea are well preserved (probably as the result of the extirpation of the uterus).

In a few other cases in which apparently the stage of heat is approaching, we find good intermediate or even proliferating glands. Apparently the same factors which prepare uterus and ovaries for heat, exert also some effect on the mammary gland. Notwithstanding the presence of good deciduomata and of mature follicles, the gland may still be resting in certain other cases. In one the ovaries were hypotypical, a condition which explains the inactivity of the mammary gland which is accompanied by an inactive state of the uterus.



*Mammary Gland from the Beginning of the 20th to the 27th Day.*—This period does not occur under normal conditions. Under normal conditions a new ovulation usually precedes this period. But if through incisions in the uterus, made 4 to 8 days after ovulation, deciduomata are experimentally produced, a new ovulation is often delayed, and the sexual period prolonged. The mammary glands of twenty-four animals were examined during this period; of these fifteen were resting glands, five were proliferating, and four were intermediate. The five proliferating glands were found in animals 21 to 25 days after previous ovulation. In the first animal the thyroid had been entirely or almost entirely extirpated. Corpora lutea as well as mature follicles were present. In the second incisions had been made in the uterus; the developing deciduomata had become necrotic, and the corpora lutea showed vacuolization. In this case there was little proliferation present. Proliferation in this case may have started at an earlier period, while deciduomata and corpora lutea were still in good condition. In the third animal incisions had been made in the uterus, infection of the uterus took place, and although the animal was sick, the corpora lutea were well preserved. In the fourth instance the uterus had been completely extirpated. Deciduomata were not present, but as usual under these conditions the corpora lutea were preserved and there were mature follicles present. In the fifth the corpora lutea were necrotic, but the state of the mature follicles suggested that the animal was near ovulation. In three of the guinea pigs, therefore, well preserved corpora lutea were present. Sickness of the animal due to infection of the uterus does not necessarily prevent proliferation of the mammary gland. In one of the remaining animals impending ovulation was probably responsible for the proliferation. The four animals in which one intermediate gland was found had all ovulated 21 to 23 days previously. In all these guinea pigs the corpora lutea were vacuolar, and there were in addition necrotic deciduomata. In two of the animals mature follicles were found.

The fifteen guinea pigs with resting glands or with glands slightly better than resting had ovulated during a period varying between 21 and 27 days preceding the examination. Of these the majority, namely eleven, showed degenerated corpora lutea; of the remaining



four two had good corpora lutea, in two others the corpora lutea were partly preserved; these four animals, however, had deciduomata which were entirely or partly necrotic. Of those animals which had vacuolar, retrogressing corpora lutea, five showed in addition necrotic deciduomata. Mature follicles were found in the ovaries of two of the guinea pigs, without, however, showing any indications that an ovulation was imminent.

We see then that in those animals in which there was a resting mammary gland degenerating corpora lutea were present in the majority of cases. In a few instances in which the corpora lutea were partly preserved, deciduomata were necrotic, while in two animals mature follicles were found. In none of these animals was there any indication of an imminent heat. It is also of interest to note that all the animals in which more than 23 days had elapsed since the previous ovulation, in which, therefore, the delay in ovulation was especially great, the mammary gland was in a more or less resting stage. These facts are of interest in considering the succeeding set of animals.

*Mammary Gland in Guinea Pigs in Which Living Deciduomata and Well Preserved Corpora Lutea Are Present.*

It was advisable to make a separate group of these animals in order to determine whether the presence of experimentally produced deciduomata without the accompaniment of embryonic structures, together with the presence of good corpora lutea, affect the mammary gland. It was especially of interest to compare these animals with others of the corresponding period of the sexual cycle in which pregnancy was found.

We examined the mammary gland in twenty-five animals belonging to this group. Twenty-one of the animals have not been included in the various groups of the normal sexual cycle; in these ovulation had occurred later than 16 days previous to examination, while the four animals in which ovulation had occurred from 12 to 16 days previously have been referred to above.

*Mammary Gland from the 9th to the End of the 13th Day of the Sexual Cycle.*—Seven animals belong to this class. One had a proliferating

gland; it has been mentioned among the animals in the normal sexual cycle. Two animals, both of which were sick, had a resting gland. Of four in which the gland was intermediate, one showed mature follicles. We see, therefore, that at this stage of the cycle the glands as a rule show no proliferation notwithstanding the presence of good deciduomata and well developed corpora lutea.

*Mammary Gland from the Beginning of the 14th to the End of the 17th Day.*—In three animals the mammary gland was proliferating. In two the corpora lutea showed a slight peripheral vacuolization, and in the third the corpus luteum was good throughout. In two of these three animals mature follicles were present. Three animals had an intermediate gland. In one of these the corpora lutea showed the beginning of vacuolization. In another the ovaries had not been examined, but there is little doubt that the corpora lutea were at this period still fairly well preserved. Mature follicles were also present in one animal. One-half the guinea pigs, therefore, had a proliferating, while the remainder showed an intermediate gland.

*Mammary Gland from the Beginning of the 18th to the End of the 20th Day.*—All three animals showed a proliferating gland. The corpora lutea were well preserved, and mature follicles were present in all of them.

If we combine the second and third groups, we have nine guinea pigs of which two-thirds show a proliferating, and the remaining one-third an intermediate gland. If we compare this result with the condition in animals of the normal cycle without the presence of good deciduomata, and in approximately the corresponding period of the sexual cycle, we find much greater activity in the mammary gland of animals in which good deciduomata are present. Without good deciduomata during an approximately corresponding period we find the majority of glands in a resting condition and only a little more than one-third proliferating.

*Mammary Gland from the Beginning of the 21st to the End of the 23rd Day.*—Nine animals were examined at this period. In six with good deciduomata and corpora lutea which were well preserved or showed just beginning peripheral vacuolization, the mammary gland was proliferating. In all but one of these animals mature follicles were present in the ovaries. In one animal with good corpora lutea

and with deciduomata that showed slight degeneration, it was doubtful whether the mammary gland was proliferating or intermediate. In this case also mature follicles were present. In the last two guinea pigs in which there were also good deciduomata and good corpora lutea present, the mammary gland was in one case intermediate, in the other not quite intermediate.

*Mammary Gland from the Beginning of the 24th to the Beginning of the 27th Day.*—In all these four animals good deciduomata and corpora lutea that were well preserved or showed merely beginning peripheral vacuolization were present. All had mature follicles. Three showed a proliferating gland, one a gland that was better than intermediate.

From the 21st to the 27th day the mammary glands of thirteen animals were examined. Nine of these were definitely proliferating, one perhaps proliferating, and the remaining three were intermediate. The majority were, therefore, proliferating; none were resting in this group. If we compare these glands with the mammary glands of animals of the corresponding period, in which the deciduomata were mostly necrotic, we find a great difference. In the latter the majority were in a resting condition. It is probable that both preservation of corpora lutea as well as deciduomata contributed to this result. In the former group of corresponding age in several instances we found a good corpus luteum without deciduomata, combined with a proliferating gland. However, in cases in which necrotic deciduomata were present, the mammary gland was usually resting, while in this group with good deciduomata the mammary gland was usually proliferating. We may then conclude that in cases in which the sexual cycle is artificially prolonged, well preserved deciduomata are usually associated with proliferating glands, while degenerating deciduomata which are usually combined with vacuolar corpora lutea, are associated with resting glands. Good corpora lutea and good deciduomata may, therefore, at this period of the sexual cycle cause proliferation of the mammary gland without the presence of pregnancy.

Mature follicles, such as are found even in cases in which vacuolar corpora lutea and necrotic deciduomata are present, are not able to cause proliferation in the mammary gland. Even at an earlier date, in the period from the 16th day on, the presence of good cor-

pora lutea combined with good deciduomata in the majority of cases produced proliferation of the mammary gland.

The period from the 9th to the 14th day of the sexual cycle belongs to the refractory period of the sexual cycle. At this time even the presence of good corpora lutea combined with well preserved deciduomata could only in one case produce proliferation; but in the period from the 14th to the end of the 24th day of the sexual period, there were fifteen or sixteen proliferating glands and six which were intermediate. It is probable that the deciduomata contribute to the effect of the corpus luteum. In three out of four cases in which good corpora lutea were present, but the deciduomata necrotic, the mammary gland was resting, while in the fourth case it was proliferating notwithstanding the necrotic condition of the deciduoma and a somewhat vacuolar state of the corpus luteum.

We see then that a definite cycle of the mammary gland corresponds to the cycle in the ovary and uterus. In the first period we find proliferation of the mammary gland, which gradually diminishes as the sexual cycle progresses, then comes a resting period at a time when the corpus luteum is well developed and functioning, to be followed again by beginning proliferation at a period when the new sexual cycle in the ovary and uterus is approaching. If we prevent the onset of the new sexual cycle and artificially prolong the old cycle, then we find that proliferation of the mammary gland is usually associated with the presence of good deciduomata and corpora lutea, while in the presence of necrotic deciduomata, which are often but not always accompanied by vacuolar corpora lutea, the mammary gland usually remains resting.

#### *Effect on the Mammary Gland of Extirpation of the Ovaries.*

In nineteen guinea pigs both ovaries were extirpated,—in the majority of instances 5 to 7 days, in some, however, as early as 3 days after ovulation. In all but two incisions were made in the uterus at the time of castration. The development of deciduomata was interfered with; they either did not develop at all or remained small and had become necrotic, if the examination took place 20 days after ovulation or later. In the two animals in which no incisions had

been made in the uterus, the examination was made approximately 18 days after ovulation; the mammary gland was resting or intermediate. In one case in which incisions in the uterus had been made, on examination 14 days after ovulation, the mammary gland proved to be resting or slightly better than resting. In this guinea pig little deciduomatous tissue had been produced.

In another in which the examination took place 9 days after ovulation the gland was almost resting. In a third animal 14 to 15 days after ovulation a small deciduoma had developed which had become entirely necrotic and showed many hemorrhagic areas. The mammary gland was resting to intermediate. In a fourth and fifth instance at the examination  $9\frac{1}{2}$  to  $10\frac{1}{2}$  days after ovulation, the mammary gland was likewise resting to intermediate. In these five guinea pigs the mammary glands were, therefore, examined during the negative period. Proliferation could not be expected at that period even with the presence of normally functioning ovaries. In the other twelve cases examination took place 20 to 22 days after ovulation. In almost all, deciduomata had developed which had become necrotic. The mammary gland varied in these cases between the resting and intermediate condition.

As we have shown, in the presence of good corpora lutea or of a combination of good corpora lutea and good deciduomata, proliferation of the mammary gland is frequent between the 18th and 22nd days after ovulation. After extirpation of the ovaries, however, proliferation was absent during this period. We may therefore conclude that extirpation of the ovaries prevents proliferation of the mammary gland. After castration those influences emanating from the uterus and ovaries which, under certain conditions, cause a proliferation of the mammary gland, are absent. It may be that the same condition which leads to the early necrosis of the deciduomata after castration also prevents proliferation of the mammary gland. In addition, the absence of good deciduomata or the presence of necrotic deciduomata may in such cases have an additional unfavorable influence on the mammary gland.



*Mammary Gland in Animals with Hypotypical Ovaries.*

Loeb has formerly described a condition of the ovaries in which the follicles do not reach full size.<sup>3</sup> Atresia sets in at a stage when the follicles are as yet of medium size or even smaller. Such ovaries he designated as hypotypical. It seems that in almost all these cases corpora lutea are either degenerating or absent. We observed ten cases of this kind in which the condition of the mammary gland was ascertained. While in the majority of the animals there can be no doubt about the character of the ovaries, it is possible that in an exceptional case an accidental transitory condition was included in this series. In all these guinea pigs the mammary gland was resting or only slightly better.

In one of the animals the ovaries were examined 6 days after ovulation. The corpora lutea had been extirpated 4 days previously. In the second case incisions had been made in the uterus and only the remnants of degenerated corpora lutea were visible. The third guinea pig died 13 days after heat; the animal was in a state of inanition; the corpora lutea showed beginning vacuolization. In the fourth animal a few small pieces had been cut out from the ovaries 5 days after ovulation; 22 days after ovulation retrogressing vacuolar corpora lutea were found. In the fifth the thyroids had been (perhaps incompletely) removed  $4\frac{1}{2}$  days after ovulation;  $23\frac{1}{2}$  days after ovulation vacuolar corpora lutea were found. In the sixth instance, 26 days after thyroidectomy, very much degenerated remnants of corpora lutea were found. In the seventh guinea pig the thyroids had been extirpated  $5\frac{1}{2}$  days after ovulation. On examination 23 days later degenerating corpora lutea were found; no new ovulation had taken place. In both the sixth and seventh cases the animals had been sick before examination of the ovaries. In the eighth case small pieces had been excised from the ovaries almost 4 days after ovulation. 10 days later the guinea pig died. A large, well preserved, corpus luteum was present. In the ninth animal blood had been withdrawn on two occasions, and 3 days after the last bleeding the animal was killed. Only the remnants of corpora lutea were present. In the last case an incomplete extirpation of corpora lutea

<sup>3</sup> Loeb, *Zentr. Physiol.*, 1911-12, xxv, 342.



had been done 7 days after ovulation; 10 days later the examination showed the presence of vacuolar, small corpora lutea. A new ovulation had not taken place in many of these cases.

We find, then, that hypotypical ovaries are combined with an inactive state of the mammary gland and that guinea pigs with hypotypical ovaries behave like castrated guinea pigs.

*Effect of Extirpation of the Corpora Lutea on the Mammary Gland.*

We stated above that in cases in which the ovaries were hypotypical the corpora lutea were usually absent or degenerating, and that in these cases the mammary gland was inactive. A similar condition of the mammary gland is observed in cases in which at an early stage of the sexual cycle the corpora lutea have been extirpated without a new ovulation taking place. As one of us has shown previously, extirpation of the corpora lutea leads in the majority of cases to a hastening of the new ovulation which marks the onset of a new sexual cycle.<sup>4</sup>

We examined the mammary gland in thirty-three guinea pigs in which the corpora lutea had been completely extirpated. We may divide this set into two groups: in the first one, comprising thirteen animals, a new ovulation had not yet taken place at the time of examination; one of these animals has already been mentioned among the guinea pigs in which the ovaries were hypotypical. In the second group, comprising twenty animals, a new ovulation had occurred at the time of examination.

(a) In the first group we found a resting mammary gland in six animals which were examined at a period from 4 to 17 days after ovulation. In these animals the corpora lutea had been absent from 2 to 11 days. In one of these cases incisions had been made into the uterus 6 days after ovulation; on examination 10 days later, small living deciduomata were found. The examination in these six guinea pigs was made at a time, when, during the normal cycle, no proliferation usually occurs in the mammary gland.

In seven instances the mammary gland was intermediate. In four of the animals the examination took place at an early period of the

<sup>4</sup> Loeb, *Deutsch. med. Woch.*, 1911, xxxvii, 17.

sexual cycle; namely, from 5 to 9 days after the ovulation which preceded the extirpation of the corpora lutea. In these four the corpora lutea had been absent from 3 to 7 days. In the remaining three cases incisions had been made into the uterus, in two 6 days, and in one  $3\frac{1}{2}$  days after ovulation. In two of the animals no deciduomata developed owing to the absence of the corpora lutea, and in the third they had become necrotic at the time of examination. Examination was made in these cases  $17\frac{1}{2}$  days after the ovulation which preceded the extirpation of the corpora lutea. The corpora lutea had been absent 13 or 14 days. In two of these guinea pigs mature follicles were present and one of them was in heat. Notwithstanding the presence of heat, this animal, in which deciduomata had not developed in the cut uterus, did not show a proliferating gland. In the third animal, in which we were only able to examine one ovary, it is doubtful whether a new ovulation had not taken place. We find, therefore, after extirpation of the corpora lutea an intermediate gland either in animals with mature follicles or in animals examined during the first half of the sexual cycle, the latter class representing cases in which the period of absence of corpora lutea had been relatively short. It is probable that considering the occasional presence of proliferation in the mammary gland during the corresponding period of the normal cycle and its complete absence after extirpation of the corpora lutea, that the absence of corpora lutea is partly responsible for the non-proliferating condition in the mammary gland. On the other hand, we have seen that if heat or ovulation occurs, proliferation of the mammary gland sets in during the normal cycle notwithstanding the absence of good corpora lutea. This latter conclusion is confirmed by experiments in which after extirpation of the corpora lutea, a premature new ovulation took place.

(b) The second set comprises twenty animals in which after extirpation of the corpora lutea, a new ovulation was imminent or had taken place. As stated above, after complete extirpation of the corpora lutea the new ovulation is generally accelerated.

Two animals were found near or during the stage of heat. In one of these in which the uterus had not been cut, the mammary gland was proliferating. In this case the preceding heat had occurred a little over 12 days previously, and the corpora lutea had been

absent 10 days. The second animal has already been mentioned under (a). In this case incisions had been made in the uterus, but no deciduomata developed. Examination was made  $17\frac{1}{2}$  days after ovulation; corpora lutea had been absent for 14 days. The mammary gland was almost intermediate. In three cases the animals were near ovulation or ovulation had taken place within the preceding 24 hours. No incisions had been made into the uterus. Corpora lutea had been absent in these animals for 8, 9, and 14 days, respectively, and extirpation of the corpora lutea had taken place from 2 to 3 days following ovulation. In two of the animals the mammary gland was proliferating, in the third its structure was almost that of a proliferating gland, but mitoses were not found. In three animals examination took place from 1 to 3 days after ovulation. Here the second ovulation took place from 11 to 15 days after the first one, and corpora lutea had been absent during a period varying from 7 to 9 days. In all these animals the mammary gland was proliferating.

In six guinea pigs in which the ovulation following extirpation of the corpora lutea had taken place 3 to 5 days, and in seven other guinea pigs in which the second ovulation had taken place  $5\frac{1}{2}$  to 6 days previous to examination, the mammary gland was found in an approximately intermediate condition. In most of these animals extirpation of the corpora lutea, which was complete in each animal, as microscopic examination showed, had been carried out 2 to 3 days after the first observed ovulation. Examination took place 11 to 15 days after extirpation of the corpora lutea and the ovulation following extirpation of the corpora lutea was accelerated in all cases. In a number of these cases incisions had been made in the uterus, but deciduomata did not usually develop. Only in animals in which the corpora lutea had been extirpated relatively late, namely,  $6\frac{1}{2}$  days after ovulation, did deciduomata develop; in one of these they had become necrotic, in the second small, good deciduomata were visible at the time of examination.

These experiments confirm the conclusion at which we arrived through our observations of the normal cycle; namely, that in the guinea pig the stage of heat and ovulation and the period immediately succeeding it are accompanied by a proliferation of the mammary gland, while the later period, coincident with the development

of the corpus luteum, is not accompanied by a proliferation. We learn, furthermore, that an absence of corpora lutea during a certain period preceding heat and ovulation does not only not prevent subsequent proliferation of the gland, but is a prerequisite for this proliferation, inasmuch as without a preceding degeneration or extirpation of all the corpora lutea no new heat or ovulation can take place. This conclusion is in accordance with the fact previously demonstrated by one of us that corpora lutea, probably through chemical influence, inhibit heat and ovulation.<sup>4</sup> In a similar manner the corpora lutea inhibit that proliferation of the mammary gland which is characteristic of the first period of the sexual cycle. It is probable that this particular influence of the corpora lutea on the mammary gland is not a direct, but an indirect one, the direct action being exerted on the sexual cycle of ovaries and uterus, which act on the mammary gland.

While extirpation of the ovaries prevents the onset of a new sexual cycle and therefore the concomitant proliferation of the mammary gland, extirpation of the corpora lutea hastens both of these processes. Another constituent, namely, in all probability the follicular apparatus or part of it, is responsible for the occurrence of heat, ovulation, and the proliferation of the mammary gland in the early period of the sexual cycle.

#### *Effect of Extirpation of the Uterus on the Mammary Gland.*

Loeb has previously observed that extirpation of the uterus seems to have a preserving influence on the corpora lutea.<sup>5</sup> It is therefore of interest to contrast specially this group of experiments with the preceding group in which the corpora lutea were absent. In four animals the mammary gland had been examined after an extirpation of the uterus, which had been carried out at an early period of the sexual cycle. Examination took place in one case 17, in two cases 18, and in the fourth case 23 days after the preceding ovulation. In two small deciduomata were present at the lower stumps of the uterus. Excision of the uterus had, therefore, not been complete.

<sup>5</sup> It is intended to investigate further this relationship between extirpation of the uterus and the life of the corpora lutea.

In the remaining cases deciduomata were not found. In all four instances the corpora lutea were well preserved and the mammary gland was proliferating. It is probable that the proliferation of the mammary gland and the preservation of the corpora lutea in these cases stand in a causal relation to each other.

#### SUMMARY.

1. A definite cycle exists in the mammary gland of the non-pregnant guinea pig which corresponds to the cycle in the ovary and uterus. This cycle can be presented through a curve in which the ordinates represent the degree of activity of the gland in a series of animals, and the abscissæ the time since ovulation (period of sexual cycle). The curve passes through a first maximum at the time of heat and ovulation and gradually falls. The minimum is reached on the 6th day and continues until the 15th day after ovulation. Next begins the period when a new ovulation is imminent and the number of the proliferating glands again increases. We see then that during the normal cycle the presence of well preserved, functioning corpora lutea does not lead to proliferation, neither do mature follicles have such an effect. On the other hand, the absence or degeneration of the corpora lutea is required to insure the proliferation of the mammary gland in the first period of the sexual cycle.

If the sexual period is experimentally prolonged, we find in some instances proliferation, while in others it is absent. As far as we can determine at the present time, two factors seem to favor proliferation of the mammary gland under these conditions: (1) the presence of well preserved corpora lutea, particularly if they are associated with well preserved experimentally produced deciduomata, and (2) the imminence of a new period of heat. The connection between good corpora lutea and good deciduomata and the presence of proliferating mammary glands at this stage of the sexual cycle is, however, not absolute. There are cases in which a proliferating gland is associated with some degeneration of the corpus luteum. Or on the other hand a well preserved corpus luteum is associated with a non-proliferating gland. In some of the latter cases the simultaneous presence of a necrotic deciduoma may perhaps explain the lack of proliferation in the mammary gland. However, in the majority of



cases we found the presence of good corpora lutea and good deciduomata associated with a proliferating mammary gland. Whether a living corpus luteum as such is able to produce proliferation of the gland is as yet doubtful.

2. Extirpation of the ovaries prevents not only the proliferation of the mammary gland associated with the first stage of the sexual cycle, the condition of heat and ovulation no longer taking place in castrated animals, but in all probability also inhibits the proliferation of the mammary gland which occurs under certain conditions towards the end of the sexual cycle, or in instances of experimentally prolonged sexual cycle in which well preserved corpora lutea and deciduomata are present.

3. In animals in which the ovaries were hypotypical, the mammary glands were in an inactive condition. The presence of hypotypical ovaries has the same influence on the mammary gland as castration. In the majority, but not in all of these cases well preserved corpora lutea were absent.

4. Complete extirpation of the corpora lutea seems directly or indirectly to prevent the secondary proliferation of the mammary gland, which occurs during the latter part of the sexual cycle or during an experimentally prolonged cycle, in cases in which the extirpation is not followed at once by a new ovulation. This conclusion we consider, however, merely as suggested, not yet as definitely established through our results. On the other hand, the primary proliferation of the mammary gland, during the first stage of the sexual cycle, as well as ovulation and the objective signs of heat, is accelerated through complete extirpation of the corpora lutea. Thus the effect of extirpation of the corpora lutea differs from the effect of castration, in that after the latter neither a new heat nor the primary proliferation of the mammary gland occurs. As one of the authors has pointed out previously, the absence of functioning corpora lutea and the presence of either well developed ovarian follicles or of mature follicles are necessary for the occurrence of heat and ovulation. The same conditions are prerequisites for the primary proliferation of the mammary gland.

5. In cases in which the whole or almost the whole uterus had been extirpated, the corpora lutea were well preserved and the mammary gland was proliferating.



# THE CYCLIC CHANGES IN THE MAMMARY GLAND UNDER NORMAL AND PATHOLOGICAL CONDITIONS.

## II. THE CHANGES IN THE PREGNANT GUINEA PIG, THE EFFECT OF LUTEIN INJECTIONS, AND THE CORRELATION BETWEEN THE CYCLE OF THE UTERUS AND OVARIES AND THE CYCLE OF THE MAMMARY GLAND.

BY LEO LOEB, M.D., AND CORA HESSELBERG, M.D.

(From the Department of Comparative Pathology of Washington University Medical  
School, St. Louis.)

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In a previous paper<sup>1</sup> we analyzed the cyclic changes in the mammary gland in the non-pregnant guinea pig. In this communication we shall report on our experimental studies of the mammary gland in guinea pigs during or following the period of pregnancy. We shall furthermore report on the effect of injections of lutein on the mammary gland, and in conclusion shall briefly discuss the relation between the sexual cyclic changes in the ovaries and uterus on the one hand and in the mammary gland on the other.

### *Mammary Gland during Different Periods of Pregnancy.*

In twenty-six guinea pigs the mammary gland was examined during different periods of pregnancy.<sup>2</sup> Included in this series is one case of experimentally produced extra-uterine pregnancy, of which a more detailed description has been previously given by one of us<sup>3</sup> from another point of view. This series also included a case

<sup>1</sup> Loeb, L., and Hesselberg, C., *J. Exp. Med.*, 1916, xxv, 285.

<sup>2</sup> When the intervals indicated in the protocols had elapsed the guinea pigs were allowed to die under ether anesthesia, and the tissues taken out at operation were placed immediately into Zenker's fluid.

<sup>3</sup> Loeb, L., *Proc. Soc. Exp. Biol. and Med.*, 1913-14, xi, 103; *Biol. Bull.*, 1915, xxviii, 59.

in which pregnancy was present in one horn of the uterus, while in the other horn there was a beginning abortion, and a third case in which it is doubtful whether pregnancy or merely an experimental deciduoma was present. In the remaining twenty-two cases the animals had been used for certain experiments which, however, did not interfere with the progress of pregnancy, and we have every reason to assume that they did not influence the condition of the mammary gland.

In five instances the mammary gland was examined within the first 15 days after copulation. In one of these, however, the period of pregnancy may perhaps have been slightly farther advanced. In these five cases the mammary gland was found in the following condition: In one animal 6 days and in another about 11 days after copulation, the mammary gland was intermediate. In three cases the pregnancy was approximately 12 to 15 days old; in two of these cases the mammary gland was proliferating; in the third it was intermediate.

In seven guinea pigs the mammary gland was examined during a period from the beginning of the 16th to the end of the 19th day. In three of these animals the gland was proliferating, in four it was intermediate. In one of the latter cases, however, it was doubtful whether in addition to the deciduomata pregnancy was present.

In three animals the mammary gland was examined between the beginning of the 20th and the end of the 23rd day; it was found to be intermediate. There was in addition one case in which pregnancy was present in one horn and abortion was under way in the other horn 22 days after copulation. In this case the gland was proliferating.

In nine animals the pregnancy was in a further advanced stage. at a period later than 24 days after copulation; in all these guinea pigs the mammary gland was proliferating.

We see then that proliferation of the mammary gland during pregnancy becomes regular only after the 24th day, that it was absent in two cases on the 6th and 10th days; between the 12th and 24th days the gland was proliferating in five and intermediate in eight cases. In the majority, therefore, the gland is not proliferating in the early period of pregnancy.

If we compare the condition of the mammary gland during the first period of pregnancy with the corresponding period of the normal cycle not accompanied by copulation and pregnancy, we find the majority of glands in both non-proliferating. There were, however, during the normal cycle of the non-pregnant animal a considerable number of non-proliferating glands in a resting state, while during early pregnancy the non-proliferating glands were all intermediate. In this respect the mammary gland during this period of pregnancy resembles the mammary gland of non-pregnant animals of the same period in which good corpora lutea and good deciduomata were present. In this class of animals also the non-proliferative glands were not resting, but intermediate; the proportion of proliferative glands in the latter class was, however, even greater than during early pregnancy—a difference perhaps without significance and due to accident. It is probable that pregnancy as well as the presence of good deciduomata and corpora lutea improve the condition of the mammary gland even in those cases in which the stimulating effect of these conditions is not sufficiently strong to lead to proliferation. On the whole, however, in the guinea pig, pregnancy does not induce proliferation of the mammary gland to a much higher degree than the factors active during the latter part of the normal sexual cycle unaccompanied by pregnancy, and proliferation of the mammary gland during pregnancy becomes regular only at a period of time which exceeds the duration of the normal sexual cycle unaccompanied by pregnancy. In this group we may include an animal in which extra-uterine pregnancy had been produced experimentally. In this case an incision had been made into the uterus  $2\frac{1}{2}$  days after ovulation; the examination took place 18 days after the first ovulation. The condition of the ovaries indicated that about 3 days previous to the time of examination a new ovulation had taken place. The mammary gland was proliferating,—a finding not unexpected at this stage of the sexual cycle.

#### *Mammary Gland in the Period Following Pregnancy.*

In five animals the mammary gland was examined at various periods after the completion of pregnancy.

*Guinea Pig 1.*—About 12 to 24 hours after delivery. In the ovaries recently ruptured follicles with the beginning of corpora lutea are visible. The mammary gland is large and consists of many lobules with numerous acini. The acini are of larger size than in the glands described above. The epithelium is high cuboidal almost throughout; in the lumen of the acini there is a finely granular precipitate, representing the secretion of the gland. Some acini are distended with secretion; here the gland cells are flat, evidently as the result of pressure. Occasionally there are small vacuoles in that part of the cell which adjoins the lumen. In a number of acini there are within the lumen yellow bodies, round or of variable shape and size, containing small pigment granules. The nuclei of the gland cells are usually large and vesicular. A few mitoses occur in the gland cells. In some of the larger gland cells there are apparently some double nuclei present. The connective tissue between the acini shows loose texture; it is rich in fibroblasts and contains some polynuclear leukocytes and lymphocytes and plasma cells.

We find then at this stage a secreting gland in which, however, the stimulus present during pregnancy is still active and causes slight proliferation.

*Guinea Pig 2.*—15 days after delivery; new pregnancy; the animal suckles her young. Much milk is present in the gland; the acini have a rather large lumen and in some cases there is a fine, loosely arranged precipitate, and in these acini the gland cells are generally flat and vacuolar. In other acini the vacuolar cells are a little higher. The nuclei form sometimes only a narrow band around the vacuoles. Also whole gland cells, with their nuclei, apparently disintegrate in the process of milk production. Some acini show irregular projections into the lumen. The acini are separated from each other by these strands of dense fibrous tissue with small nuclei. Mitoses are absent in the gland cells.

*Guinea Pig 3.*—19 days after delivery; new pregnancy; the animal suckles her young. This gland is similar to that in Guinea Pig 2, but the epithelial cells are, on the average, a little higher, especially in one acinus where no secretion is present. In some of the acini where secretion is present, some of the cells are pressed flat. Some cells apparently have two nuclei. In other cells vacuolization takes place and many cells swell and degenerate. In this case there is less connective tissue present than in Guinea Pig 2, and the connective tissue is richer in cells, which are larger and have larger vesicular nuclei. Mitoses are absent in this gland. A number of gland cells are cast off; occasionally a cast off cell has two nuclei. We find occasionally cell inclusions consisting of degenerated cells.

*Guinea Pig 4.*—29 days after delivery; new pregnancy; the animal suckles her young. This specimen represents a gland in the last period of secretion. The acini have wide lumina. Many vacuoles are visible in the gland cells. The nuclei are, on the whole, pale, and nuclear debris is seen in many acini; evidently

many cells degenerate. No mitoses can be found, but apparently a few double nuclei occur in gland cells. There is little connective tissue between the acini, but a few lymphocytes and polynuclear leukocytes are present.

We see then that after the completion of pregnancy and in the beginning of secretion, some mitotic proliferation may still be present, but that it soon ceases, probably as the result of those processes that lead to secretion. Thus while during the period of secretion, in spite of the presence of pregnancy, mitotic proliferation ceases, and the process of secretion is able to counteract the stimulating effect of pregnancy, nevertheless some proliferative stimulus appears to be active, which, however, under the conditions present during secretion, apparently merely leads to a mitotic multiplication of nuclei. This conclusion is suggested by the appearance of double nuclei in a number of gland cells. We intend, however, to investigate further the occurrence of double nuclei in secreting glands before we consider a mitotic nuclear division in secreting cells of the mammary gland as established. It also appears that during the process of secretion not only parts of the cells are transformed into material to be secreted, but that whole cells degenerate and are admixed to the secreted material.

Not only during proliferation, but also during secretion the formation of a large quantity of dense fibrous tissue is prevented in the mammary gland.

*Guinea Pig 5.*—25 days after delivery; new pregnancy; the animal does not suckle her young. The gland is large, but the acini are small, and they have only small lumina. The epithelium is cuboidal. In some of the larger acini some colloid-like material is present. No mitoses are seen. The connective tissue between the acini is highly cellular; dense fibrous tissue has not been produced. Some polynuclear leukocytes are present in the interstitial tissue. This gland is, therefore, still in an intermediate condition, but might have been expected within a short time to begin to proliferate.

#### *Mammary Gland after Abortion.*

In eight animals the mammary gland was examined soon after abortion had begun or had been completed. In all these cases some experimental procedure had taken place previously, which probably led directly or indirectly to the abortion.



In five instances the abortion took place between the 17th and 22nd days of pregnancy. In these cases the mammary gland did not present the picture of a secreting gland, but was either resting, intermediate, or proliferating. In two cases abortion took place in the latter part of pregnancy, 49 to 51 days after copulation. In guinea pigs the normal duration of pregnancy is approximately 65 to 66 days. In these cases the abortion produced secretion in the mammary gland, and microscopically the typical picture of secretion was present. We may therefore conclude that after proliferation has proceeded for a certain time, inhibition of the proliferative stimulus of pregnancy leads to such changes, of a more or less degenerative character in the gland cells, as precede and cause secretion. One of the animals (No. 6) again demonstrates the fact observed after the normal completion of pregnancy that at an early period of secretion there may still be present some cell proliferation in the secreting mammary gland.

The eight cases observed are briefly as follows:

*Guinea Pig 7.*—After incomplete extirpation of the corpora lutea, abortion took place 18 days after copulation; resting gland.

*Guinea Pig 8.*—Cuts had been made into the ovaries; abortion took place 19 days after last ovulation; resting gland.

*Guinea Pig 9.*—After incomplete extirpation of the corpora lutea, abortion had taken place 17½ days after previous ovulation; partly proliferating gland.

*Guinea Pig 10.*—After thyroidectomy and incomplete extirpation of the corpora lutea, abortion took place. Examination 22 days after last ovulation; intermediate gland.

*Guinea Pig 11.*—In a similar instance in which the mammary gland was partly proliferating, abortion had taken place only in one horn, while the other was pregnant. This case will be referred to below.

*Guinea Pig 12.*—Two intravenous injections of hirudin had been given to a pregnant guinea pig; beginning abortion; a proliferating gland was found. In this case the stage of pregnancy at the time of abortion is not certain.

*Guinea Pig 6.*—On the 49th day of pregnancy cuts were made into a mammary gland. Abortion took place. The gland was proliferating as well as secreting. Frequent mitoses were found in those areas in which there was no secretion in the acini; where there was some secretion, mitoses were still present; mitoses were seen even in acini much distended by secretion. In different lobules the degree of secretion and proliferation varied. The connective tissue was rather cellular; some polynuclear leukocytes and lymphocytes were present in the stroma. It is possible that the incisions in the uterus, made previous to abortion, were



in this case responsible for the mitotic proliferation; perhaps the stimulus supplied by pregnancy caused it.

*Guinea Pig 13.*—A large secreting gland without mitoses; abortion was beginning on the 41st day of pregnancy.

*Effect on the Mammary Gland of Extirpation of the Ovaries during Pregnancy.*

The mammary gland was examined in four guinea pigs in which the ovaries had been extirpated  $3\frac{1}{2}$  to  $6\frac{1}{2}$  days after copulation, and in which notwithstanding the absence of the ovaries, the pregnancy continued for some time. The mammary gland was examined  $17\frac{1}{2}$  to  $18\frac{1}{2}$  days after copulation. In three cases there was beginning abortion; in one of these abortion existed as yet only in one horn of the uterus, while in the other horn pregnancy still proceeded. In the fourth animal, in which the ovaries had been extirpated  $5\frac{1}{2}$  days after copulation, a normal pregnancy was still present 13 days after castration. In these four instances the gland was either resting or intermediate. Proliferation was absent. From these data we can draw no definite conclusions as to the direct effect of placenta or embryo on the mammary gland, because during the stage of pregnancy at which examination took place, proliferation in the mammary gland is often absent even in normal animals.

*Mammary Gland in Pregnant Animals after Extirpation of the Corpora Lutea.*

In seventeen animals the corpora lutea were extirpated in most cases about 6 days, in some cases 3 to 4 days after copulation. Pregnancy proceeded usually until the time of examination, which took place 14 to  $18\frac{1}{2}$  days after copulation; in some cases abortion had taken place previous to, or was proceeding at the time of the examination. We can arrange these seventeen cases into two classes according to whether or not a new ovulation had taken place at the time of examination. In the first class (*a*) comprising seven animals no new ovulation had taken place; in the second class (*b*) comprising ten animals, a new ovulation had taken place at the time of examination.

*Animals in Which a New Ovulation Had Not Taken Place at the Time of Examination.*—In three of these cases pregnancy was still present from  $15\frac{1}{2}$  to  $18\frac{1}{2}$  days after copulation, and after an absence of the corpora lutea during a period of 9 and 12 days. In three other cases abortion was just taking place, and in the last animal abortion had been completed at the time of examination. In three animals the mammary gland was intermediate, in three others not quite intermediate, and in one the mammary gland was somewhat better than intermediate, but not proliferating. In this case almost mature follicles were present in the ovaries, and it is possible that a new period of heat was impending.

*Animals in Which a New Ovulation Had Taken Place at the Time of Examination.*—In three guinea pigs new ovulation had taken place within 2 days preceding examination, a period of the sexual cycle during which proliferation is usually present in the mammary gland. In the first one of these animals ovulation had taken place sometime between 6 and 18 hours previously; abortion was just proceeding; the mammary gland was in part proliferating. In the second abortion had been completed at the time of examination. A new ovulation had occurred about  $1\frac{1}{2}$  days preceding examination. In this guinea pig it is not certain that mitoses were present in the mammary gland, but the structure of the gland indicated that some proliferation might have been present a short time previously. In the third case pregnancy was still present, ovulation had taken place about 2 days before examination; the mammary gland was proliferating. In these three animals the corpora lutea had been absent during a period varying between 8 and 11 days. These observations confirm conclusions formulated in the preceding paper. The period directly following ovulation is accompanied by proliferative changes in the mammary gland, even if the ovulation has been accelerated experimentally, with or without a concomitant pregnancy. These observations furthermore prove that abortion does not necessarily prevent these proliferative changes.

In seven other guinea pigs ovulation had taken place about 3 to 8 days preceding examination. All were still pregnant. In one case, examined 19 days and a few hours after copulation, incisions had been made into one horn of the uterus at the time of the extirpation

of the corpora lutea. Small deciduomata developed, of which one was still alive and one necrotic at the time of examination. A new ovulation had taken place about 7 days previously and corpora lutea had been absent during a period of 6 days. In this animal the mammary gland was slightly proliferating. In the other six the mammary gland was either resting or resting to intermediate. In these instances the pregnancy was 18 to 19 days old, and the corpora lutea had been absent during a period varying between 6 and 9 days. In four of these animals a new ovulation had taken place 3 to 6 days previous to examination; the mammary gland was resting to intermediate. In two cases a new ovulation had taken place 6 to 8 days previously; in these two animals the mammary gland was in a resting stage.

We see, therefore, that the same factors determine conditions in the mammary gland if cyclic changes have been induced experimentally during pregnancy and in cases in which the normal cyclic changes have taken place without pregnancy.

If we consider the cases in which, during pregnancy, the corpora lutea or ovaries had been extirpated and examination was carried out 15 to 20 days after the last ovulation, we find that in none of the animals examined was the gland proliferating, while during the corresponding stages of either the ordinary cycle without pregnancy or during the corresponding period of pregnancy, a certain proportion of glands was found proliferating. This makes it probable that in this case also extirpation of the corpora lutea or ovaries directly or indirectly inhibited proliferative changes in the mammary gland. Extirpation of the corpora lutea during pregnancy, however, does not prevent, but on the contrary accelerates the proliferative changes in the mammary gland concomitant with a new ovulation.

#### *Effect of Injections of Lutein on the Mammary Gland.*

It seemed important to test the effect of the corpus luteum on the mammary gland in still another way; namely, directly through injections of the corpus luteum substance into the peritoneal cavity of the guinea pig.<sup>4</sup> 0.5 to 1 gm. of lutein was suspended in a sterile

<sup>4</sup> For this purpose we used Armour and Company's preparation of lutein from the cow.

bottle in 10 to 12 cc. of distilled, sterilized water and shaken in a mixing apparatus during a period varying between  $2\frac{1}{2}$  and 7 hours. The fluid was then centrifuged for a few minutes and thus the undissolved residue removed. The supernatant fluid was injected intraperitoneally; about 8 to 12 cc. of fluid were given each time to one animal. Twenty-two guinea pigs were thus injected and an interval of 2 or 3 days was allowed to elapse between two succeeding injections. In seven of the animals the ovaries had been extirpated, in eight the corpora lutea had been removed, in one of the latter incompletely. In four animals incisions had been made in the uterus; similar incisions had been made in the uterus in animals in which ovaries or corpora lutea had been extirpated. In three cases the injections were made during the second half of the sexual cycle without any other experimental interference with the animal. In a few instances in which incisions had been made in the uterus previous to the injections, smaller or greater parts of the uterus became infected as the result of the injections. Some bacteria were apparently present in the lutein specimens. After injections into an uninjured peritoneal cavity, they did not lead to infection, but sometimes they infected the surface of a cut uterus. Lutein injections as such did not produce proliferation of the mammary gland, although sometimes as many as five injections were given to the same animal.

In seven cases lutein was injected in animals in which both ovaries had been extirpated; at the same time incisions were made into the uterus. Two of the animals received five, one three, two two injections, and two one injection. In a few animals the first injection of lutein was given 1 day before castration. The animals were examined 11 to 15 days after the preceding heat. In none was proliferation noticeable in the mammary gland, which was either resting or varying between the resting and intermediate state.

Injections of lutein were given to eight animals in which the corpora lutea had been extirpated. In one the extirpation had been incomplete. In one case five, in two cases four, in one case three injections of lutein were given, and in three cases one injection was given. In several guinea pigs the first injection was given 1 or 2 days before the operation. In the majority of the animals incisions had been made in the uterus at the time of extirpation of the cor-

pora lutea; in two of these an infection of the cut uterus occurred, evidently as the result of the lutein injections. In these eight animals the examination took place at the following periods.

(1) 17 days after the preceding heat; five injections had been given; the mammary gland was resting. (2) 7 days and 17 hours after the preceding heat; extirpation was incomplete; one injection had been given; the mammary gland was intermediate. (3)  $13\frac{1}{2}$  days after the previous heat; four injections had been given; the mammary gland was resting. (4)  $16\frac{1}{2}$  days after the preceding heat; four injections had been given; the mammary gland was approximately resting. (5) 6 days after the preceding heat; 3 days after the lutein injection, one injection being given; the gland was intermediate. (6) 5 days after the preceding heat and 2 days after the lutein injection, one injection being given; the mammary gland was intermediate. (7)  $10\frac{1}{2}$  days after the preceding heat; small deciduomata were slightly infected at some places; one injection had been given; the mammary gland was resting. (8) 14 days after the preceding heat; three injections were given; the mammary gland was almost resting.

We see that the injections of lutein did not exert any noticeable influence on the mammary gland in animals in which the corpora lutea had been extirpated.

In four animals incisions in the uterus were made some time previous to the injections of lutein.

(1) Incisions were made in the uterus during the positive period (3 to 7 days after ovulation).  $17\frac{1}{2}$  days after heat the first injection of lutein was made;  $26\frac{1}{2}$  days after heat examination took place. Good deciduomata had developed, the corpora lutea were preserved. Four injections of lutein were given. The mammary gland was resting. (2)  $6\frac{1}{2}$  days after ovulation incisions were made into the uterus.  $18\frac{1}{2}$  days after ovulation one injection of lutein was given. Examination of the animal took place  $21\frac{1}{2}$  days after ovulation. The uterus, in which deciduomata had developed, was markedly infected; no mature follicles, but good corpora lutea were present in the ovaries; the mammary gland was proliferating. (3) Five injections of lutein were given;  $25\frac{1}{2}$  days after ovulation examination was made. Large deciduomata and corpora lutea which were on the whole preserved, but showed some peripheral vacuolization, were present. A new ovulation had not taken place. The mammary gland was intermediate. (4) Four injections of lutein were given; examination  $26\frac{1}{2}$  days after ovulation. 11 days after ovulation the first injection of lutein was given. Living deciduomata and small corpora lutea were found. The mammary gland was proliferating.

We find proliferating mammary glands in several cases of this series. But inasmuch as this might be expected even without lutein injections, we cannot ascribe this result to the injection of lutein.



In three cases injections of lutein were made in animals in which no experimental interference had taken place. The injections were made in the latter half of the sexual cycle.

(1) Five injections were given, the first one  $13\frac{1}{2}$  and the last  $22\frac{1}{2}$  days after heat; examination took place  $23\frac{1}{2}$  days after heat. No new ovulation had occurred. The mammary gland was resting. (2) Four injections were given, the first 15 and the last 22 days after heat; examination was made 24 days after heat. A new ovulation had occurred about 4 days before examination. The mammary gland was found to be intermediate. (3) Four injections were given, the first  $12\frac{1}{2}$  and the last  $19\frac{1}{2}$  days after heat;  $23\frac{1}{2}$  days after ovulation examination was made. A new ovulation had taken place 5 to 6 days previous to examination. The mammary gland was intermediate.

In one control case four injections of 14 cc. of 0.4 per cent sodium chloride solution were made intraperitoneally, the first injection being given  $11\frac{1}{2}$  days and the last  $21\frac{1}{2}$  days after ovulation; examination 22 days after the first heat showed that a new ovulation had occurred  $2\frac{1}{2}$  to 3 days previous to examination. The mammary gland was therefore found to be partly proliferating.

In these cases again results were obtained which had to be solely attributed to the condition of the sexual cycle. The mammary gland was apparently not influenced by the injection of lutein. We may then conclude that as many as five consecutive injections of lutein given to the same guinea pig do not exert a noticeable stimulating effect on the mammary gland. While it is possible that a still larger number might have produced a definite effect, we should have expected to notice microscopically at least a beginning proliferation after as many as five injections. But in no case could such an effect be established. Under normal conditions the action of the corpus luteum is probably continuous; under the conditions of our experiment the action was not continuous, although a relatively large quantity had been injected each time.

It is, furthermore, possible that the effective constituent of the corpus luteum possesses a certain species specificity, and that while the corpus luteum extract of the cow is ineffective, the extract of the guinea pig corpus luteum might have been effective. Of course we have to consider the fact that even during the normal cycle proliferation in the mammary gland takes place in the absence of the



corpus luteum and that on the other hand proliferation is absent at certain periods when corpora lutea are well developed. In this respect, then, the negative result of the injections of lutein agrees with what we found at certain periods in the normal animal. However, we found indications that the corpus luteum may favor proliferation of the mammary gland under certain conditions, and our experiments prove that as many as five injections of cow's lutein are unable to produce even the beginning of proliferation.

#### SUMMARY.

1. In the pregnant guinea pig proliferation of the mammary gland becomes regular only at a later stage of pregnancy; namely, during the period following the 24th day of pregnancy. Previous to this period proliferation was absent in the majority of cases. Proliferation of the mammary gland during pregnancy becomes regular only at a period of time which exceeds the duration of the normal sexual cycle unaccompanied by pregnancy. It is probable that pregnancy as well as the presence of living deciduomata and corpora lutea increases the proliferative activity of the mammary gland as compared with the ordinary cycle in non-pregnant animals or in animals lacking corpora lutea and deciduomata.

2. After the completion of pregnancy and in the beginning of secretion some mitotic proliferation may still be present, but it soon ceases, probably as the result of those processes that lead to secretion. While during the period of secretion, notwithstanding the presence of a new pregnancy, mitotic proliferation soon ceases, some proliferative stimulus seems still to be active, which, however, under existing conditions apparently leads only to a mitotic multiplication of nuclei. The latter conclusion is only suggested at the present time and needs confirmation through further studies.

3. In cases in which abortion took place in the first half of pregnancy secretion in the gland was not established; secretion occurred in two animals aborting toward the latter part of pregnancy. In one of these cases, mitotic proliferation of some gland cells was associated with the microscopic appearances of secretion.

4. In guinea pigs castrated during an early period of pregnancy

in which pregnancy continued for some time, proliferative changes were absent in the mammary gland. In conjunction with a partial similar effect observed after extirpation of the corpora lutea during pregnancy, we may perhaps attribute the lack of proliferation in some of these cases to the absence of the ovaries.

5. Extirpation of the corpora lutea during pregnancy induces a new ovulation and with it the primary proliferation in the mammary gland; abortion does not necessarily prevent these proliferative changes. Extirpation of the corpora lutea during pregnancy perhaps prevents the secondary proliferative changes in the mammary gland.

6. Five injections of cow's lutein given in relatively large quantities intraperitoneally do not produce proliferation of the mammary gland in the guinea pig.

*Correlation of the Cyclic Changes in the Uterus and Ovaries with Those in the Mammary Gland.*

We can distinguish two phases in the cycle of the ovaries and uterus; the first phase comprises the period of heat, ovulation, and the period immediately following ovulation. In this period certain growth processes take place in the uterus. The processes taking place in the uterus and ovaries during this period are dependent on the removal of an inhibiting substance produced by the corpus luteum and on the presence of a substance produced through another constituent of the ovaries, in all probability the large follicles including the mature follicles or perhaps the latter alone. As we pointed out previously, an interstitial gland comparable to that of the rabbit does not exist in the guinea pig. Heat changes therefore depend on the absence of the corpus luteum and on the presence of another ovarian constituent.

This period is followed by the second and longer period dominated by the function of the corpus luteum. As we have shown experimentally, the secretion of the corpus luteum sensitizes the uterine mucosa in such a way that the decidual and predecidual proliferation takes place, the former after application of mechanical stimuli or after the injury produced through the embryo penetrating into the mucosa, the latter under the influences of ordinary metabolic

changes. This sensitizing influence of the corpus luteum is, however, not exerted throughout the whole remaining part of the sexual cycle, but only during a period of 6 or 7 days (from the 3rd to the 9th or 10th day after ovulation). We must either assume that the corpus luteum ceases to secrete this sensitizing substance after the 9th day after ovulation or else that after this period it secretes in addition a substance inhibiting the action of the first substance. We have shown that the corpus luteum has an inhibiting action by force of which it prevents the appearance of a new ovulation and heat. This latter substance is given off during the whole period of the sexual cycle. It is therefore probable that we have to deal with two different substances secreted by the corpus luteum.

During pregnancy conditions are essentially the same as without pregnancy. The corpus luteum exerts the same two functions. There is only one essential difference between the conditions during pregnancy and without pregnancy. If we accelerate experimentally the onset of a new sexual cycle without pregnancy, the newly formed corpus luteum causes also a new cycle in the uterine mucosa, while during pregnancy the experimental production of a new corpus luteum is not followed by a new cycle in the uterine mucosa; here some inhibiting mechanism is at work. If we now correlate the cyclic changes in the mammary gland with those in the uterus and ovaries, we find proliferative changes in the mammary gland corresponding to the first phase of the ovarian and uterine cycle. We may call this the primary growth period of the mammary gland. We have every reason to believe that the swelling of the human mammary gland during menstruation, observed by many and in the absence of microscopic studies referred to vascular changes, represents in part these growth processes.

We are able to accelerate experimentally the primary proliferation of the mammary gland; in the same way we can experimentally accelerate heat and ovulation, through removal of the inhibiting tonus exerted chemically by the corpus luteum. The primary growth period of the mammary gland can be experimentally induced as well during pregnancy as without pregnancy. Contrary to what might have been expected, we find that the growth stimulus exerted on the uterus by the corpus luteum in the first part of the second phase of

the sexual cycle does not find a counterpart in the mammary gland of the guinea pig. During the whole period of the development and activity of the corpus luteum of the non-pregnant animal the mammary gland in the majority of cases is inactive. Only during the last part of the sexual cycle may we find in some cases some proliferation of the mammary gland. Whether this is due to the approach of the succeeding period of heat or is an accumulative late effect of the corpus luteum substance cannot be determined with certainty at present. It is possible that a quantitatively stronger action of the corpus luteum at last produces proliferative changes also in the guinea pig. Thus we usually found proliferation in cases in which we artificially prolonged the period of the sexual cycle and in which well preserved corpora lutea and deciduomata were present. How much in these cases the proliferation of the mammary gland has to be attributed to a quantitatively stronger action of the corpus luteum or to the deciduoma or to the combination of both, cannot be decided definitely at the present time.

The conditions are similar during pregnancy. We find here definite growth of the mammary gland only after the passing of a period which is longer than the duration of the normal sexual period. But in a number of instances we find following the 12th day of pregnancy some proliferation in the mammary gland. Pregnancy as well as the presence of living deciduomata and well preserved corpora lutea causes an increase in the number of proliferating glands during a time corresponding to the closing period of the normal sexual cycle. We might designate this as the secondary growth period of the mammary gland. In the pregnant animal it develops into the typical hypertrophy of the mammary gland characteristic of this condition, and, indeed, represents merely its initial stage.

In the rabbit Ancel and Bouin<sup>5</sup> as well as Frank and Unger<sup>6</sup> noticed a parallelism between the presence of good corpora lutea and the

<sup>5</sup> Ancel, P., and Bouin, P., *J. physiol. et path. gén.*, 1911, xiii, 31.

<sup>6</sup> Frank, R. T., and Unger, A., *Arch. Int. Med.*, 1911, vii, 812. Shortly after the preliminary communication of our results (*Proc. Soc. Exp. Biol. and Med.*, 1915-16, xiii, 91) there appeared a preliminary note by M. Athias (*Compt. rend. Soc. biol.*, 1916, lxxix, 557) in which this author described growth and secretion in the mammary gland in castrated male guinea pigs, into which ovaries had been transplanted. At the time of examination, soon after the beginning of lactation,



growth of the mammary gland. Ancel and Bouin conclude that it is the corpus luteum which determines the growth of the mammary gland. Our investigations show that in the guinea pig the factors determining the growth of the mammary gland are complex, and that they can be considered only partly analyzed at the present time. Provided the facts observed by Ancel and Bouin in the rabbit have found a correct interpretation, we must assume that in the rabbit the response of the mammary gland to the secretions of the corpus luteum is much more prompt than in the guinea pig. We would have to assume that the gland cells in the rabbit are more labile and thus react at once, while in the guinea pig the stimulus has to accumulate during a considerable period of time before a reaction sets in. From a biological point of view such a difference between the rabbit and guinea pig could well be understood if we consider the fact that in the rabbit the duration of pregnancy is much shorter than in the guinea pig, and that therefore the service of the mammary gland is required in the rabbit at a much earlier period after copulation than in the guinea pig. While such a consideration is not a causal explanation, it renders it at least plausible that a marked difference in the power of reaction of the two kinds of mammary gland does exist.

As to the secretory function of the mammary gland, we are inclined to consider it as a phenomenon intermediate between growth on the one hand and complete degeneration on the other hand, as we find it with deciduomata and under certain conditions also in the mammary gland<sup>7</sup> after the growth stimuli have suddenly ceased to act. It seems that the regenerating effect of all divisions is necessary in certain tissues in order to insure their continued preservation. The peculiar constitution of the mammary gland causes instead of complete degeneration, an intermediate state of intercellular degeneration, which is, however, coupled with a degeneration of a considerable number of gland cells. Such a view is somewhat akin to that of Hildebrandt<sup>7</sup> without, however, being identical with the latter.

there were no well developed corpora lutea in the ovaries. The author concludes that follicles or interstitial gland, but not the corpora lutea are responsible for the growth of the mammary gland. (It is not our intention to consider extensively the literature on this subject at the present time; we may give a more complete review at a later date.)

<sup>7</sup> Hildebrandt, P., *Beitr. chem. Phys. u. Path.*, 1904, v, 463.





# A COMPARATIVE STUDY OF CERTAIN ACTIONS OF ADRENALIN IN THE CAT AND THE RABBIT.

By THOMAS STOTESBURY GITHENS, M.D.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

PLATES 29 TO 31.

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After unilateral excision of the superior cervical ganglion, certain substances or conditions produce an effect on the side on which the ganglion has been removed, while on the other side on which nothing has been done, or on which the sympathetic nerve has been cut below the ganglion, the pupil is affected but little or remains comparatively unaffected. This is called paradoxical pupil dilatation.

Meltzer and Auer<sup>1</sup> found that subcutaneous injection or instillation in the conjunctival sac, which has no effect upon the pupil of the normal rabbit, produces considerable dilatation of the pupil in the rabbit on the side on which the superior cervical ganglion has been removed, while the pupil on the side on which only the sympathetic nerve has been cut, remains unchanged; in other words, adrenalin causes a paradoxical pupil dilatation in the rabbit. Meltzer<sup>2</sup> found later that the same holds true for the cat, but only when adrenalin is administered by subcutaneous injection. He has not been successful with instillation of adrenalin into the eyes of operated cats, on account, he says, of the "protruding nictitating membrane which would sweep over the entire eye as soon as adrenalin came in contact with it, thus removing the adrenalin." Meltzer adds that he has not been persistent enough in his attempts.

In a series of experiments on cats we have made many attempts to produce dilatation by instillation of adrenalin in the conjunctival sac on the side on which the ganglion was previously removed and have found that it is difficult to produce dilatation in the cat by this method. While in rabbits dilatation of the pupil is readily obtained

<sup>1</sup> Meltzer, S. J., and Auer, C. M., *Am. J. Physiol.*, 1904, xi, 28.

<sup>2</sup> Meltzer, S. J., *Am. J. Physiol.*, 1904, xi, 37.

by a single instillation, in cats we obtained in many cases a moderate dilatation only after many instillations in the course of 2 hours, and in many instances we failed. In addition to the action of the nictitating membrane mentioned by Meltzer, it is possible that the conjunctiva of the cat is more dense and does not favor absorption.

However, it seemed doubtful that these mechanical factors alone could sufficiently account for the failure of conjunctival instillation, in view of the ease with which eserine and atropine cause dilatation of the pupil in the cat. There seemed to be no obvious reason why adrenalin should be absorbed less readily than these substances, and we thought that there might be other reasons for the difference in the response of the two species of animals.

Our first problem was to establish whether the responsiveness of the iris differed in the two species, and we believed that some light might be thrown on the matter by studying the effect of adrenalin, given by intravenous injection, on the cat's pupil and comparing this effect with that of similar injections in the rabbit. By this means all differences in the degree of conjunctival absorption would be eliminated.

The solution used for the injection was made by dissolving adrenalin crystals in 0.7 per cent sodium chloride with hydrochloric acid, and diluting until an accurate 0.1 per cent solution was obtained. For the smaller doses this was further diluted before injection. In this way we had a reliable solution at our disposal throughout the experiments.

All the animals used had undergone excision of the superior cervical ganglion, at least several days, and often several weeks before the experiments. Notes were made on the pupil of the intact, as well as on that of the operated side.

The rabbits were either held gently without narcosis and the injection was given into the marginal vein of the ear, or the jugular vein was exposed after anesthetizing the skin with ethyl chloride and the injection given into the vein. No difference was observed between the results of injections into the jugular and the ear vein. Cats were always injected in the jugular vein.

There is some difficulty in obtaining records with cats, on account of the paradoxical dilatation which appears so readily in excited

animals after gangliectomy. If the cat is stroked gently during the operation, it becomes quiet and the pupils return to normal. If the needle is then inserted in the vein and a few seconds are permitted to elapse before the injection is given, no dilatation results from the manipulation alone, as we have observed repeatedly by injection of saline solution. In a few instances the adrenalin caused marked disturbance of heart action with dyspnea. The results of these injections were discarded.

The present study deals exclusively with the size of the pupil; other effects of adrenalin on the eye were disregarded in the protocols.

The response of the pupil to intravenous injection of adrenalin has been studied in the cat by Lewandowsky<sup>3</sup> and in the rabbit by Joseph.<sup>4</sup> Lewandowsky made only qualitative observations and gives no figures either as to dosage of the adrenal extract or the degree of dilatation of the pupil which it produces. Joseph studied the quantitative response of the pupil of the rabbit after gangliectomy, but his observations were limited to the operated side. In the main, our results agree with Joseph's, the only difference being that in our experiments the response of the rabbit's pupil to a given dose was greater, both in degree and duration.

Our results may be briefly stated as follows: In the rabbit the dilating influence of intravenous injections of adrenalin was not seen in the eye on the unoperated side with doses smaller than 0.05 mg. per kilo of body weight; with doses less than 0.1 mg. per kilo the effect was not constant and when manifest was slight and lasted only a few minutes. A dose of 0.1 mg. per kilo of body weight caused, as a rule, moderate or slight dilatation with return to normal in about 10 minutes. In two animals no dilatation was seen on the intact side from this quantity. Dilatation of the pupil of the gangliectomized side was noted with doses of 0.01 mg. per kilo of body weight. With doses below 0.02 mg. however, the effect was slight and inconstant. The most constant results were obtained with a dose of 0.1 mg. With this dose a maximal dilatation ensued which lasted about 10 minutes, followed by a gradual return to normal in the course of 2 or 3 hours.

<sup>3</sup> Lewandowsky, M., *Arch. f. Physiol.*, 1899, 360.

<sup>4</sup> Joseph, D. R., *J. Exp. Med.*, 1912, xv, 644.

In the cat the response was found to be much greater. On the eye of the intact side doses of adrenalin as small as 0.01 mg. per kilo of body weight gave a distinct and constant effect, and a dose of 0.1 mg. gave rise to almost maximal dilatation. The duration was, however, brief even with the largest dose, the pupil becoming normal within 5 minutes. Similar relations were found on the operated side. Here minute doses, even as small as 0.002 mg. per kilo of body weight, caused a distinct dilatation, and 0.01 mg. per kilo gave rise to almost maximal dilatation. In both cases the dilatation was transient. A dose of 0.1 mg. per kilo of body weight caused maximal dilatation lasting about 5 minutes with a gradual return to normal within half an hour.

#### ILLUSTRATIVE PROTOCOLS.

*Rabbit 1.*—White female; weight 1.480 kilos.

Jan. 21, 1916. Right superior cervical ganglion excised under ether anesthesia.

Mar. 29, 9.21 a.m. Injected in ear vein, 1 cc. of adrenalin, 1 : 50,000, (0.0135 mg. per kilo).

Before injection,	right pupil	5 mm. in diameter;	left 5 mm.
$\frac{1}{2}$ min. after injection,	" "	8 " " "	" 5 "
$1\frac{1}{2}$ " " "	" "	8 " " "	" 5 "
2 " " "	" "	7 " " "	" $4\frac{1}{2}$ "
4 " " "	" "	$5\frac{1}{2}$ " " "	" $4\frac{1}{2}$ "
5 " " "	" "	5 " " "	" 5 "

Apr. 6, 10.00 a.m. Injected intravenously 0.15 cc. of adrenalin, 1 : 1,000 (0.1 mg. per kilo).

Before injection,	right pupil	5 mm. in diameter;	left 5 mm.
$\frac{1}{2}$ min. after injection,	" "	9 " " "	" 6 "
3 " " "	" "	9 " " "	" 6 "
5 " " "	" "	8 " " "	" 3 "
10 " " "	" "	$6\frac{1}{2}$ " " "	" 4 "
15 " " "	" "	7 " " "	" 5 "

*Rabbit 2.*—White female; weight 1.910 kilos.

Jan. 21, 1916. Right superior cervical ganglion excised under ether anesthesia.

Apr. 5, 3.23 p.m. Injected intravenously 0.19 cc. of adrenalin, 1 : 1,000 (0.1 mg. per kilo).

Before injection,	right pupil	4 mm. in diameter;	left 4 mm.
$\frac{1}{2}$ min. after injection,	" "	$8\frac{1}{2}$ " " "	" $3\frac{1}{2}$ "
1 " " "	" "	8 " " "	" $3\frac{1}{2}$ "
3 " " "	" "	8 " " "	" 5 "

10	min. after injection, right pupil	10 mm. in diameter; left 3 mm.
17	" " " " " "	10 " " " " 4 "
57	" " " " " "	8½ " " " " 3 "
77	" " " " " "	6½ " " " " 3 "
100	" " " " " "	5½ " " " " 4 "
112	" " " " " "	4 " " " " 2 "

*Cat 1.*—Black male; weight 2.660 kilos.

Mar. 27, 1916. Left superior cervical ganglion excised under ether anesthesia.

Mar. 30, 10.13 a.m. Injected intravenously 2 cc. of adrenalin, 1 : 50,000 (0.014 mg. per kilo).

	Before injection,	left pupil a slit;		right a slit.
	$\frac{1}{2}$ min. after injection,	" " 10 mm. in diameter;	"	4 mm.
1	" " "	" " 6 " " "	"	2 "
2	" " "	" " 1 " " "	"	1 "

10.23 a.m. Second injection. 2 cc. of adrenalin intravenously, 1 : 50,000 (0.014 mg. per kilo).

	Before injection,	left pupil	1 mm. in diameter; right	1 mm.
	$\frac{1}{2}$ min. after injection,	" "	10 " " "	2 "
1	" "	" "	8 " " "	1 "
2	" "	" "	2 " " "	slit.
7	" "	" "	1 " " "	1 mm.

10.45 a.m. Third injection. 2 cc. of adrenalin intravenously, 1 : 10,000 (0.07 mg. per kilo).

	Before injection,	left pupil	1 mm. in diameter; right	1 mm.
	$\frac{1}{2}$ min. after injection,	" "	15 " " "	10 "
1	" "	" "	15 " " "	8 "
2	" "	" "	12 " " "	6 "
3	" "	" "	12 " " "	2 "
4	" "	" "	10 " " "	1 "
6	" "	" "	6 " " "	2 "

Apr. 10. Weight unchanged.

10.09 a.m. Injected intravenously 0.285 cc. of adrenalin, 1 : 1,000 (0.11 mg. per kilo).

Before injection,	left pupil	3 mm. in diameter; right	4 mm.
$\frac{1}{2}$ min. after injection,	" "	16 " " " "	14 " "
1 " " " "	" "	16 " " " "	10 " "
$1\frac{1}{2}$ " " " "	" "	16 " " " "	6 " "
2 " " " "	" "	14 " " " "	3 " "
6 " " " "	" "	10 " " " "	3 " "
8 " " " "	" "	6 " " " "	3 " "
18 " " " "	" "	2 " " " "	2 " "

*Cat 2.*—Gray male; weight 3 kilos.

Mar. 27, 1916. Left superior cervical ganglion excised under ether anesthesia.

Apr. 10, 10.30 a.m. Injected intravenously 3 cc. of adrenalin, 1 : 1,000 (0.1 mg. per kilo).



Before injection,	left pupil	3 mm. in diameter; right 4 mm.			
$\frac{1}{2}$ min. after injection,	" "	17	"	"	12
1 " "	" "	17	"	"	$4\frac{1}{2}$
3 " "	" "	17	"	"	$2\frac{1}{2}$
5 " "	" "	17	"	"	$2\frac{1}{2}$
7 " "	" "	16	"	"	2
9 " "	" "	7	"	"	2
15 " "	" "	4	"	"	3
20 " "	" "	4	"	"	4
30 " "	" "	2	"	"	3

These examples illustrate the type of dilatation seen in the cat and in the rabbit after intravenous injections of adrenalin. The striking differences are the greater degree and briefer duration of the dilatation in the cat. This is true both for the normal and gangliectomized sides, and with all doses.

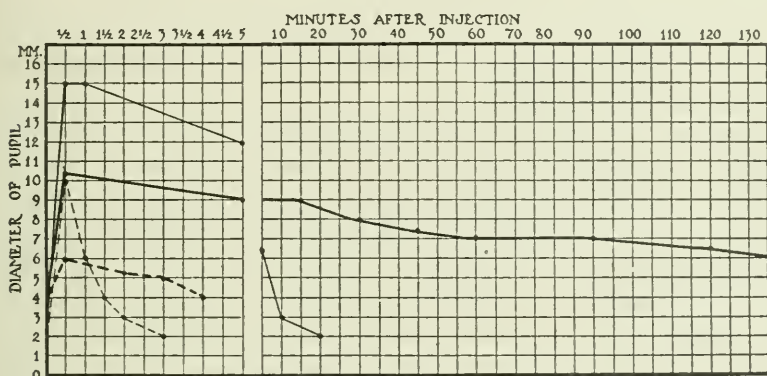
The following table shows the number of experiments and the average results.

Species.	Dose per kilo.	No. of experiments.	Pupil.	Time before return to normal.	Increase.	Maximum size of pupil.
	mg.			min.	mm.	mm.
Rabbit. ....	0.01	3	Intact side.		0	4
			Operated side.	10	2	6
Rabbit. ....	0.1	12	Intact side.	3	2	6
			Operated side.	180	6	10
Cat. ....	0.01	2	Intact side.	$1\frac{1}{2}$	7	9
			Operated side.	10	13	15
Cat. ....	0.1	13	Intact side.	2	8	10
			Operated side.	30	13	15

Text-fig. 1 shows graphically the course of the dilatation in the normal and gangliectomized pupils of rabbits and of cats, after injection of 0.1 mg. of adrenalin per kilo.

The cat's pupil, when fully dilated, is from 14 to 16 mm. in diameter, and the rabbit's from 10 to 12 mm. It will be seen that in the cat the pupil on the operated side showed approximately complete dilatation from even the smaller dose, while the rabbit showed full dilatation with the larger dose, but not even approximating it with

the smaller. The pupil of the normal side of the cat also showed a greater response to adrenalin than that of the rabbit. Comparison of the increase in diameter of the pupil of the intact side with that of the operated side, in each species, might seem to show a greater increase in response following gangliectomy in the rabbit, but this is deceptive and due to the marked effect on the pupil of the unoperated side of the cat, which would render a dilatation three times as great, such as occurs in the rabbit, an impossibility.



TEXT-FIG. 1. The course of dilatation of the pupil after intravenous injection of 0.1 mg. of adrenalin per kilo. Broken lines indicate the intact side; unbroken, after gangliectomy. Light lines show the course in cats; heavy lines, in rabbits.

Our original problem, namely, the question whether the difference in response of the two species to instillation of adrenalin was dependent on a greater responsiveness of the iris of the rabbit, seems, therefore, to be satisfactorily answered. Our experiments seem to show definitely that this is not the case. With intravenous injections it was found that the opposite was true; that is, that the responsiveness of the iris of the cat was greater than that of the rabbit. In other words, a given dose of adrenalin, injected intravenously, will cause wider dilatation of the pupil in the cat than in the rabbit.

The experiments brought out, however, the fact that while the degree of dilatation of the pupil which is brought about by a given dose of adrenalin is greater in the cat than in the rabbit, the duration

of the dilatation is, on the contrary, longer in the rabbit than in the cat. The question arose whether these inverse relations are confined to the iris, or whether it would be met also in the various other effects of adrenalin on the functions of the body.

The most readily observed and best known action of adrenalin is its constricting power on the arterioles. We therefore made a study of the influence of intravenous injections of adrenalin on the blood pressure in the cat and the rabbit, comparing the intensity of the reaction with the duration of the rise in the two species of animals.

The technique in this series of experiments differed from that already described in that all the animals were kept under light narcosis throughout the experiments.

The results showed that with all doses from 0.01 to 0.3 mg. the average effect upon the blood pressure was greater in the rabbits than in the cats, both in regard to the height and the duration of the rise. The actual height reached was greater in the cats, as their original blood pressure was higher, varying from 100 to 110 mm., while in the rabbits the normal blood pressure varied from 60 to 70 mm. With 0.1 mg. of adrenalin per kilo of body weight there was in rabbits an average rise of 85 mm. (actual maximum 150 mm.) with a return to normal in about 6 minutes (Fig. 1); in cats the rise was about 75 mm. (actual maximum 175 mm.) lasting about  $3\frac{1}{2}$  minutes (Fig. 2). With 0.03 mg. per kilo, rabbits showed a rise of 70 mm. (maximum 135 mm.) lasting 4 minutes (Fig. 3); cats showed a rise of 45 mm. (maximum 150 mm.) lasting  $3\frac{1}{2}$  minutes (Fig. 4). With 0.01 mg. rabbits showed a rise of 55 mm. (maximum 120 mm.) lasting 3 minutes; cats showed a rise of 35 mm. (maximum 140 mm.) lasting 2 minutes.<sup>5</sup>

It will be seen that the influence of adrenalin on blood pressure differs from that on the iris. In the iris the duration of the effect was at variance with the degree of dilatation, the one being greater in the rabbit the other in the cat. With blood pressure we find that the two differ in the same direction, both the duration and intensity of the effect being greater in the rabbit.

<sup>5</sup> In the rabbits the return to the base-line signaled the end of the response. In more than half the cats the return to the base-line was followed by a fall of from 10 to 40 mm. below the line with a gradual return to normal in from 2 to 10 minutes.

## DISCUSSION.

The original problem was to determine whether there were other explanations than that suggested by Meltzer to account for the difference of response of the iris of the cat and rabbit to instillations of adrenalin. One possibility was that the iris of the rabbit was more sensitive to this substance. The experiments with intravenous injections showed, however, that the opposite was the case; namely, that the iris of the cat is more sensitive to adrenalin than that of the rabbit.

In these experiments it was, however, found that with regard to the pupil there was a contrast between responsiveness and duration. While in the cat the responsiveness was greater than in the rabbit, the duration of the effect was greater in the rabbit than in the cat. This led to the investigation of the effect of adrenalin on blood pressure in the two species to determine whether it showed the same relation. Here it was found on the contrary, that the responsiveness of the vasoconstrictors as well as the duration of the response was greater in the rabbit than in the cat.

It is possible that the brevity of the dilating effect upon the cat's pupil, seen in the intravenous injections of adrenalin, is an additional factor in the very slight effect of adrenalin on the pupil of these animals, when administered by instillation. The slight absorption which perhaps actually takes place produces no perceptible effect on account of the lack of a cumulative action.

## SUMMARY.

The present investigation brought out the facts that the pupil of the cat is little affected by instillation of adrenalin, but shows, nevertheless, a greater responsiveness to adrenalin when given intravenously than does that of the rabbit, and that the duration of the dilatation effected by intravenous injection is, on the contrary, longer in the rabbit.

In regard to the vasoconstricting effect of adrenalin when administered intravenously, it was found that the intensity as well as the duration of the rise of blood pressure was greater in the rabbit than in the cat.

## EXPLANATION OF PLATES.

Blood pressure of cats and rabbits after intravenous injection of adrenalin.

The upper line shows the blood pressure; the lower line, the base-line with the time in seconds and minutes. The numbers above the base-line show the transverse diameter of the pupil on the gangliectomized side. In Fig. 1 the upper row of numbers shows the normal eye, the lower row, the operated side.

## PLATE 29.

FIG. 1. Rabbit 4. Injection of 0.1 mg. per kilo of body weight.

## PLATE 30.

FIG. 2. Cat 4. Injection of 0.1 mg. per kilo of body weight.

## PLATE 31.

FIG. 3. Rabbit 3. Injection of 0.03 mg. per kilo of body weight.

FIG. 4. Cat 3. Injection of 0.03 mg. per kilo of body weight.



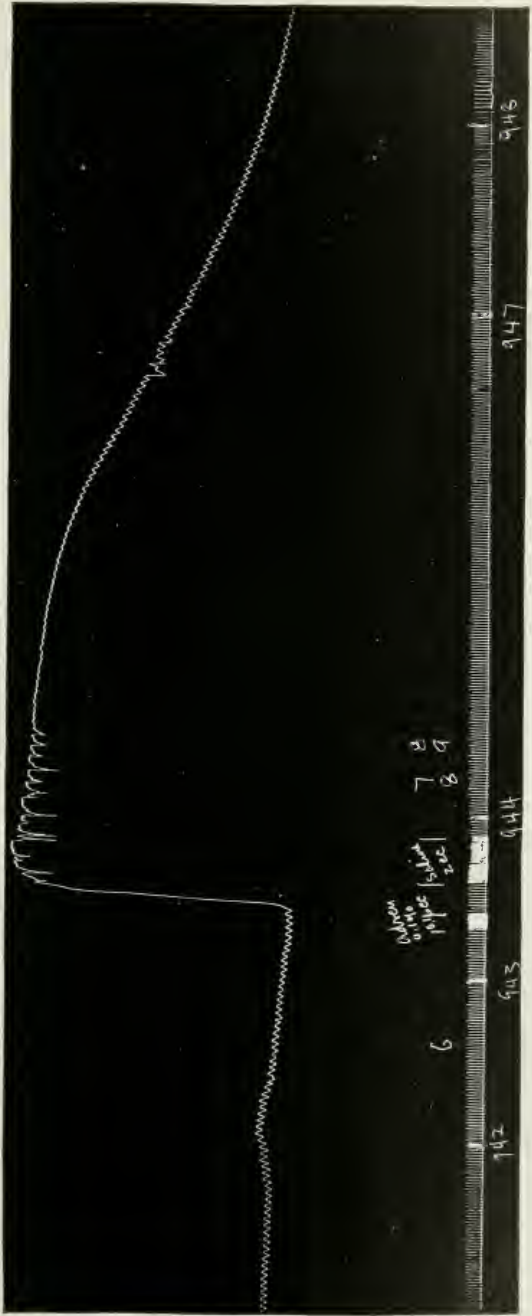


FIG. 1.

(Githens: Adrenalin in the Cat and the Rabbit.)



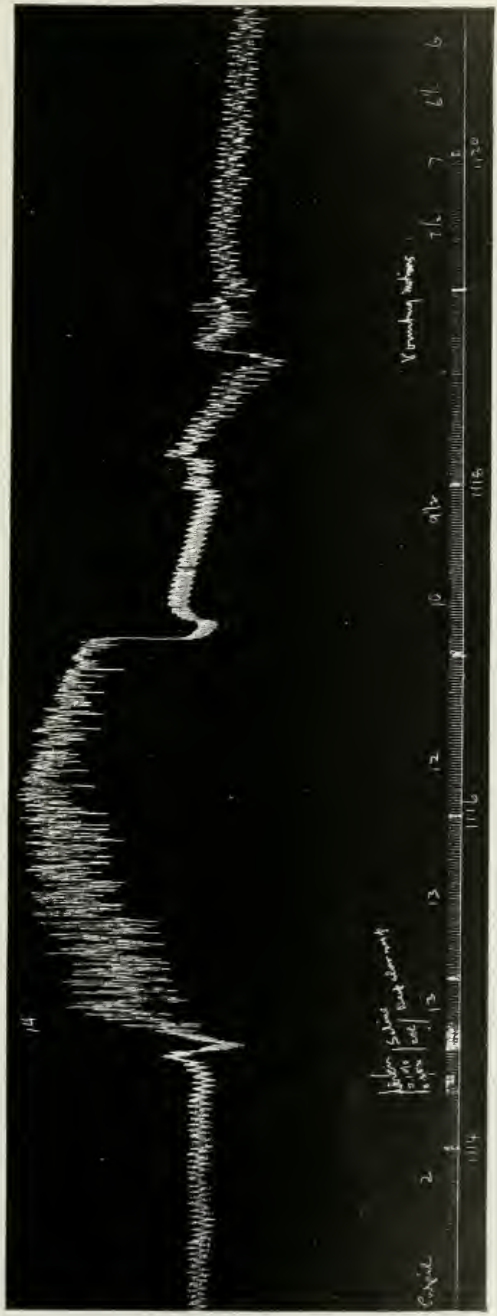


FIG. 2.

(Githens: Adrenalin in the Cat and the Rabbit.)



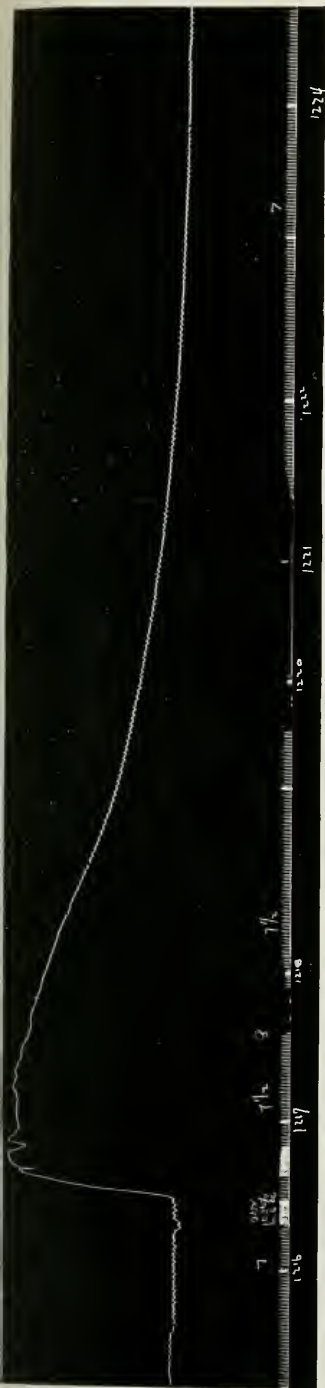


FIG. 3.

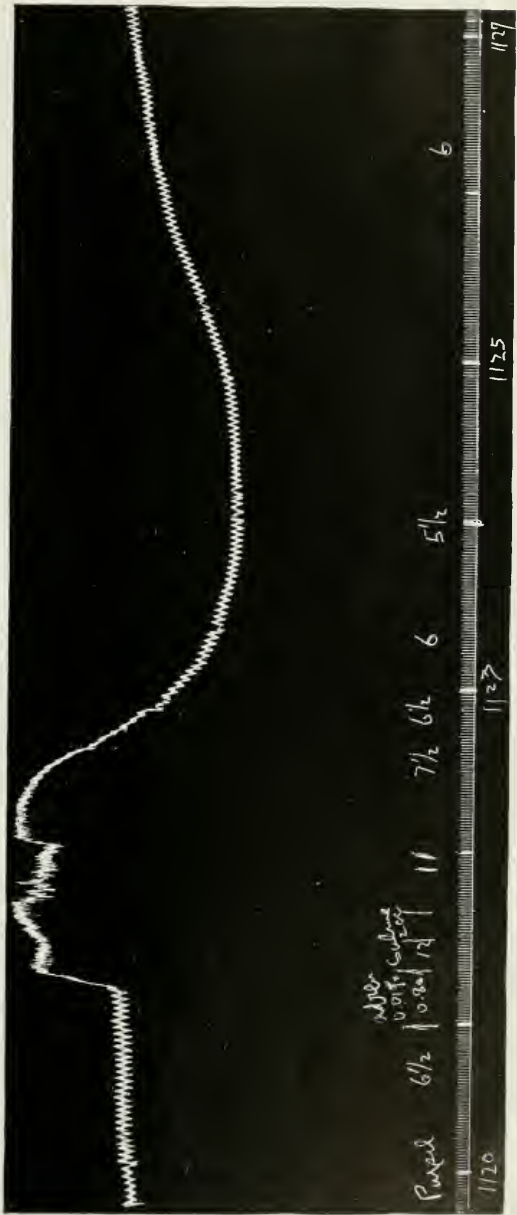


FIG. 4.





# THE SIGNIFICANCE OF THE EPITHELIAL CELLS AND SAPROPHYTES IN SPUTUM.

By MORISUKE OTANI, M.D.

(*From the Kitasato Institute for Infectious Diseases, Tokyo, Japan.*)

PLATES 32 AND 33.

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Although text-books on diagnosis state that the source of epithelial cells in the sputum may be ascertained by their microscopic appearance, the significance of this fact is not emphasized.

The mouth and pharynx are lined with polygonal flat epithelial cells, and the vocal cords are covered with them, while the nasal cavities, larynx, trachea, and bronchi are lined with ciliated cylindrical epithelial cells. The bronchioles and bronchi are lined with cuboidal cells, while the alveoli are lined with polygonal cells<sup>1</sup> which, unlike those of the other organs, have no nuclei. If inflammation occurs in any of these organs, the cells are expectorated with the mucus, and the sputum must therefore contain the epithelial cells of the affected organ. But in chronic inflammation of the larynx, as in tuberculosis, the ciliated epithelial cells lining this cavity are supplanted by a new growth of polygonal flat epithelial cells like those of the mouth. This is shown by the continual occurrence of polygonal flat cells in the sputum of laryngeal tuberculosis. The ciliated cells are distributed over a wide area, but they scarcely ever appear in the sputum. I have only once detected ciliated cells. In sputum from the lungs round or elliptical epithelial cells, which are nearly twice as large as pus cells, are often present. They are the decomposed products of the cuboidal cells found in the bronchioles. They have vesicular nuclei and contain either particles of carbon or hemosiderin in their protoplasm, and correspond to

<sup>1</sup> Some authors believe that these cells are enlarged cuboidal cells, and not epithelial cells.

Wagner's heart failure cells. I have never found flat pulmonary epithelial cells without nuclei in sputum.

The saprophytes play an important part in determining the source of sputum. In spite of the fact that various kinds of cocci, bacilli, spirochetes, and spirilla are found in the nasal and mouth cavities and in the larynx and pharynx, the sputum from the trachea and lungs is always free from these non-pathogenic microorganisms. The polygonal flat epithelial cells are found with various saprophytes, while the sputa that come from the lungs or trachea contain only the agent of the prevailing disease, such as *Bacillus tuberculosis* or *Diplococcus pneumoniae*. In cases of mixed infection, one or two species of bacteria are found. Large numbers of various kinds of microorganisms in the pulmonary or tracheal sputum were never demonstrated.

I shall refer to the sputum from the region below the vocal cords as pulmonary sputum, that from above, as laryngeal sputum, and the exudates of the nasal cavity as nasal secretions.

### *Technique.*

The same patient sometimes expectorates sputa of different appearance, revealing the various degrees of inflammation or other conditions of the affected parts. All the different sputa must therefore be examined, and the material for examination must be fresh and moist. A specimen attached to a piece of paper or gauze is unsuitable, as shown by the following experiment. A film preparation of a specimen of sputum to which some powdered starch had been added was pressed gently with a piece of blotting paper. The specimens were treated with iodine, stained with dilute fuchsin, and examined under the microscope. The particles of starch were found to be completely mixed with the sputum. On the other hand, the sputum that had been mixed with the starch suspension in salt solution and stirred vigorously proved on microscopical examination to have been mixed completely, and when subjected to the method of washing shown below, no particles of starch were demonstrated. This shows that the dehydrated starch grains stick to the sputum and cannot be washed away, while grains that have been mixed while

still moist with sputum are completely washed away. It is therefore clear that sputum from the lungs will be mixed with flat epithelial cells or some non-pathogenic microorganisms on its way to the mouth, but if the sputum is moist, the mixed elements will be completely washed away by the following method.

*Washing.*—A small specimen of the sputum is attached to the end of a platinum wire and immersed in a test-tube filled with isotonic salt solution. The material should be thoroughly rinsed with the platinum rod until it breaks up into several still smaller pieces. By washing the material once in this manner, it will usually be freed from contaminating bacteria, but sometimes the same technique must be repeated with fresh isotonic salt solution before it is cleared. The material is taken out of the salt solution, dried, and is then ready for examination.

*Film Preparation. Fresh Material.*—For the purpose of demonstrating the blood in the sputum fresh material is most suitable. The material should be washed as described above and placed on the slide. The cover-glass is then pressed lightly with the tip of the finger in order to make the film of the sputum suitable for microscopical examination.

*Stained Material.*—A small specimen of the washed sputum is put upon the middle of the slide and covered with a cover-glass, which is pressed lightly with the tip of the finger in order to obtain thin films. The cover-glass is then immediately pushed aside in contact with the surface of the slide, and removed, allowing the material to be dried in the air. If the material should be smeared with the platinum rod, the preparation is likely to be contaminated by the microorganisms attached to the rod.

*Fixation.*—For the demonstration of the blood in the sputum, the material is fixed by immersing it in absolute alcohol for 30 to 60 minutes, or in pure methyl alcohol for 15 minutes. For the examination for tubercle bacilli the material can be merely passed over the flame. Care should be taken not to let the material become too hot, as this destroys the staining properties of the pus cells and other elements.

*Staining.*—The blood is demonstrated by the same method that is employed in the preparation of the stained blood film. For the

examination of the bacilli, Ziehl's carbol-fuchsin is used, which is then decolorized with a mixture consisting of 100 cc. of absolute alcohol and two drops of highly concentrated hydrochloric acid. By this procedure, the erythrocytes lose the hemoglobin content and have the appearance of a blank vesicle. Löffler's methylene blue may be substituted by borax-methylene blue, which is prepared by mixing 1 gm. of methylene blue with 2 gm. of borax and 100 cc. of distilled water. The mixture is left standing for over 2 months and is then ready for use. It may be still further diluted to 10 per cent. It is superior to Löffler's preparation, for the cells as well as bacilli take deeper stains, while not only the original mixture, but also the dilution can be kept for a long time.

*Diagnosis.*—The most important data by which the source of the sputum can be determined are as follows: the erythrocytes or bacilli must be included in the same membrane of the mucus with other elements of the sputum such as the epithelial cells, contaminating microorganisms, or white corpuscles, which indicate the origin of the sputum itself. The diagnosis can be established only when these bodies are found closely packed in the same membrane of the mucus.

*Pulmonary Sputum.*—(Figs. 1 and 3.) Pulmonary sputum may sometimes contain round or elliptical epithelial cells a little larger than pus cells, but it never contains polygonal flat cells with nuclei. Theoretically, polygonal flat cells without nuclei may be found in the pulmonary sputum, but I have never observed any. Excepting the cases of mixed infection, one, or rarely two species of bacteria besides tubercle bacilli may be found. In no case does it contain an abundant number of different species of bacilli. If the clean sputum contains erythrocytes, the diagnosis of pulmonary hemorrhage may be made. If the same specimen should prove positive for tubercle bacilli by staining, the hemorrhage may be concluded to have occurred in the tuberculous lesions in the lungs.

*Laryngeal Sputum.*—(Figs. 2 and 4.) Laryngeal sputum usually contains polygonal flat cells with a large number of numerous species of microorganisms. It may contain only a few of these elements, but very rarely. If the laryngeal sputum contains erythrocytes the hemorrhage has occurred above the larynx. Again, if it is found to



contain tubercle bacilli, the diagnosis will be laryngeal or pharyngeal tuberculosis.

*Nasal Secretions.*—The nasal secretion of an acute nasal catarrh never contains flat epithelial cells or ciliated cylindrical epithelial cells, but may present the features of pulmonary sputum under the microscope. It usually contains, unlike the pulmonary sputum, various species of microorganisms, but sometimes contains only a few cocci or influenza bacilli. In such a case, it is difficult to establish a diagnosis by microscopical examination. In chronic nasal catarrh polygonal flat cells are always met with, but sometimes the material may contain only a few non-pathogenic contaminating bacilli. The secretion of the nasolaryngeal cavities always contains flat epithelial cells and various species of microorganisms, and presents the same features as the pharyngeal or laryngeal sputum.

By the method of examination described above, the source of the sputum can be determined and the diagnosis is thus facilitated. After several washings the material becomes free of the extraneous elements which may have come in contact with it; *e.g.*, polygonal epithelial cells. These polygonal flat epithelial cells under such circumstances will be found to be few in number, seldom exceeding two or three, and different in appearance from those of the laryngeal sputum. Moreover, in the pulmonary sputum these flat cells do not stick together with the tubercle bacilli or erythrocytes. By careful treatment and washing of the material, reliable results may be obtained. Saliva will be dissolved by the isotonic salt solution when it is washed, and completely separated from the materials together with any solid elements that might have been contained in it. If, again, two specimens of sputa are placed in contact with each other, they will not stick together unless they become dry. By washing a specimen of moist sputum, all the attached particles will be completely separated and removed from the material. By this means error in examination is avoided. If all the erythrocytes are removed by washing the material several times, the source of the hemorrhage is above the larynx.

I detected erythrocytes in the pulmonary sputum of all cases of hemoptysis examined immediately after pulmonary hemorrhage. The sputum from patients with laryngeal tuberculosis showed tubercle



bacilli in every instance. Laryngeal sputum is often so difficult to obtain that a number of examinations are usually required. Large masses of laryngeal tuberculosis sputum are seldom found; the specimens are usually small and mixed with saliva. A diagnosis, therefore, cannot always be established even after two or three examinations. From patients with both laryngeal and pulmonary tuberculosis two kinds of sputum are obtained and both must be examined. I have diagnosed the early stage of laryngeal tuberculosis in 6 out of 150 patients in whom no symptoms of the disease were evident.

Bacteriological examination of the laryngeal sputum of frank cases of laryngeal tuberculosis frequently showed tubercle bacilli; in some cases, however, scores of examinations were made before the contaminated sputum was obtained.

#### SUMMARY.

1. Microscopical examination of sputum that has been washed with isotonic salt solution indicates its source. Sputum from above the vocal cords contains polygonal flat epithelial cells and numerous species of non-pathogenic microorganisms. Sputum from below the cords is clear of saprophytes, although it sometimes contains broncho-alveolar cuboidal cells.

2. The source of the sputum can be determined by the erythrocytes and the bacilli that are contained in it, and the site of the lesion can also be ascertained.

3. In every instance I found erythrocytes in the pulmonary sputum after severe pulmonary hemorrhage.

4. Six of the cases of laryngeal affection referred to in the present paper had had no subjective symptoms, but microscopical examination showed tubercle bacilli in the laryngeal sputum. All the six cases were examined by Dr. Tanaka and were found to have tuberculous lesions in the larynx. In the laryngeal sputum of most cases of laryngeal tuberculosis with symptoms, tubercle bacilli have been frequently demonstrated, while in some cases contaminated laryngeal sputum was obtained only after a long series of examinations.

5. Microscopical examination of the sputum plays a significant part in the diagnosis of tuberculosis.

In conclusion, I wish to express my indebtedness to Dr. Tanaka, laryngologist, who supervised the laryngeal examinations for me, and to Messrs. Nemoto and Shiiba for their assistance.

#### EXPLANATION OF PLATES.

##### PLATE 32.

FIG. 1. Sputum from a patient who had a pulmonary hemorrhage (about 30 cc.); fresh specimen. Leitz oc. 1, obj.  $\frac{1}{12}$ , oil immersion. The stained preparation from the same material shows tubercle bacilli corresponding to No. V of Gaffky's scale.

FIG. 2. Laryngeal sputum from a patient with laryngitis; fresh specimen. Leitz oc. 1, obj. 4. Numerous polygonal flat epithelial cells and a few white corpuscles are seen. The stained preparation of the same material shows also numerous saprophytes. No tubercle bacilli were seen.

##### PLATE 33.

FIG. 3. Pulmonary sputum. Stained preparation. Leitz oc. 1, obj.  $\frac{1}{12}$ , oil immersion. White corpuscles, tubercle bacilli, and a cuboidal cell are seen.

FIG. 4. Sputum from a patient with laryngeal tuberculosis. Stained preparation. Leitz oc. 1, obj.  $\frac{1}{12}$ , oil immersion. White corpuscles, most of which have been destroyed, and tubercle bacilli are seen with the polygonal flat epithelial cells and numerous saprophytes, especially cocci and bacilli.



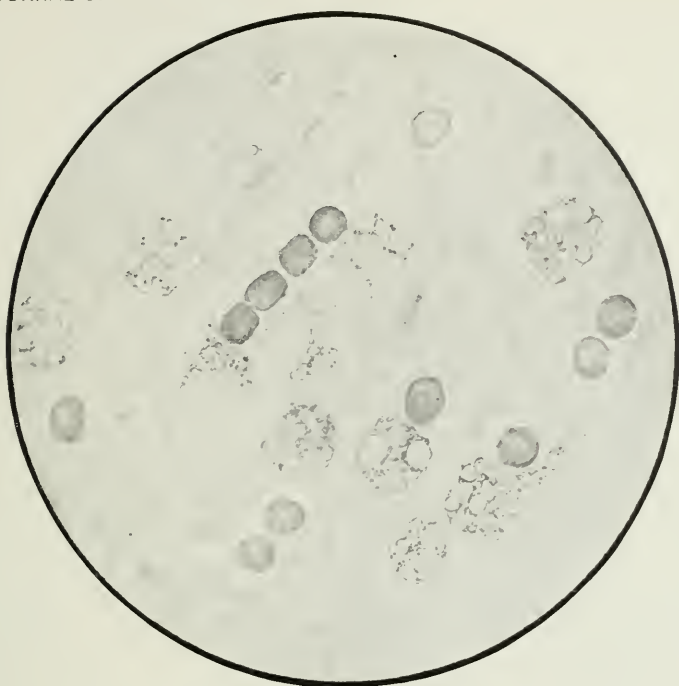


FIG. 1.

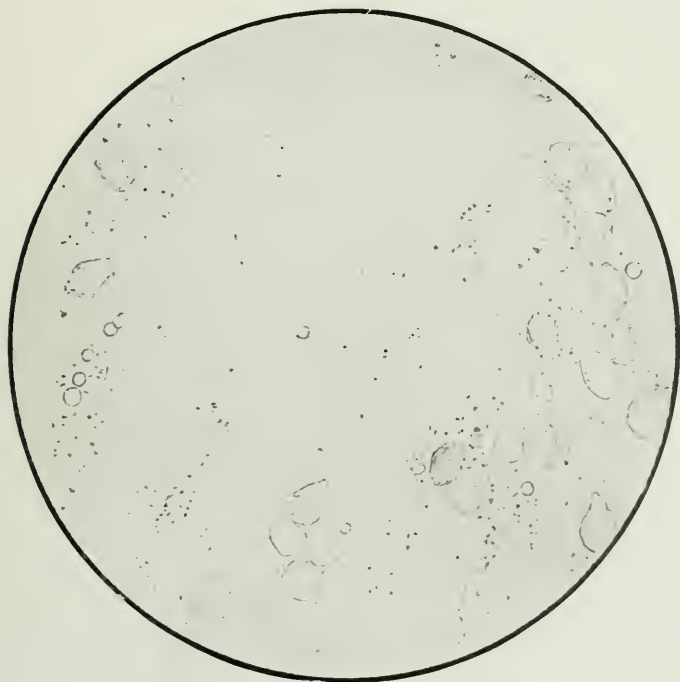


FIG. 2.

(Otani: Epithelial Cells and Saprophytes in Sputum.)



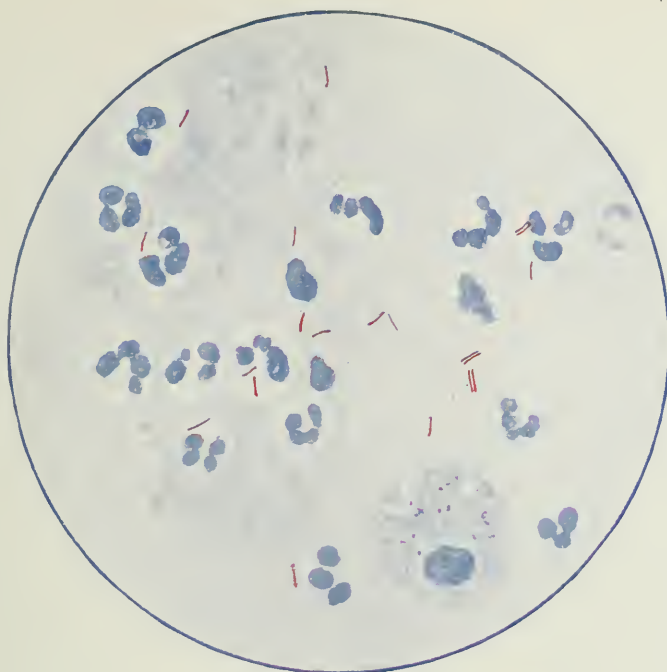


FIG. 3.

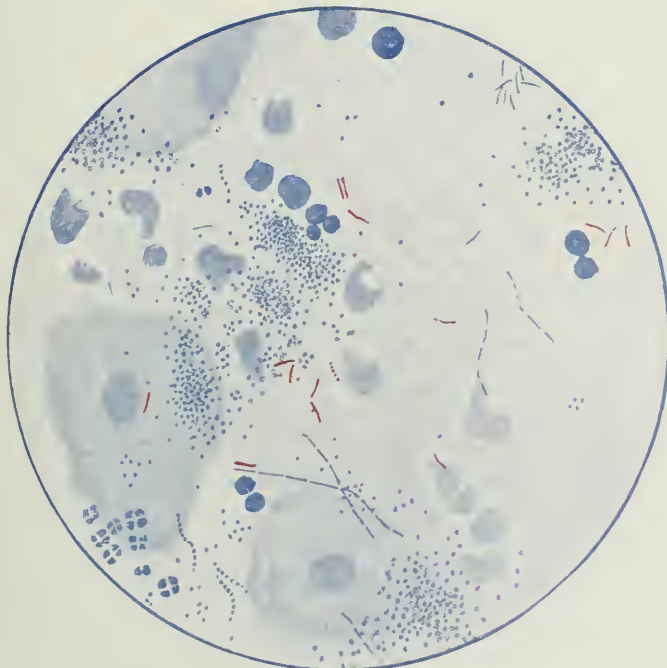


FIG. 4.

(Otani: Epithelial Cells and Saprophytes in Sputum.)





# THE CULTIVATION OF TRICHOMONAS OF THE HUMAN MOUTH (TETRATRICHOMONAS HOMINIS).

BY TOKUZO OHIRA, M.D., AND HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 34 TO 37.

(Received for publication, October 15, 1916.)

Few attempts have been made to cultivate the parasitic flagellates of man. The first report on the cultivation of the trichomonas was published by Lynch (1) in 1915.

Lynch found numerous trichomonades in the vagina and gums of a negress who was suffering from catarrhal vaginitis and acute gingivitis. He placed the material in neutral and acid bouillon and kept it alive for 3 days at 30°C. Then, as the organisms began to be affected by bacterial growth he transferred them to new media and was thus able to carry the culture through several generations.

The experiment described in this communication was carried out at first without the knowledge of Lynch's report and differs considerably from his.

*Material.*—The tartar of the mouth which has long been known to harbor trichomonades was used. In most instances they are few in number.<sup>1</sup>

*Culture Media.*—A number of different media were employed. Acid bouillon, neutral bouillon, Ringer's solution, ascitic fluid, ascitic bouillon (equal parts), ascitic fluid-isotonic salt solution (equal parts), ascitic fluid-Ringer's solution (equal parts), bouillon-Ringer's solution, sheep serum water, sheep serum water-Ringer's solution (equal parts), rabbit serum water, nutrient agar, ascitic agar, ascitic Ringer's agar, sheep serum-glucose agar. Fresh tissue was placed in both the fluid and solid media.

Most of the media were unsuitable; only a few gave relatively good results; the mixture of ascitic fluid and Ringer's solution in equal

<sup>1</sup> We wish to express here our indebtedness to Dr. M. I. Schamberg for his courtesy in providing us with some of the materials used for the present study.

portions and in its natural alkalinity gave entire satisfaction. Samples of ascitic fluid from different sources were used and gave equally good results. The bouillon employed by Lynch was not so suitable as our ascitic Ringer's solution, the chief reason probably being that it enables the contaminating bacteria to multiply so rapidly and profusely that the trichomonades no longer find it a suitable medium. Undiluted ascitic fluid was also found to be too rich in protein material, although in this respect it is more suitable than bouillon.

*Culture.*—When the culture medium is inoculated with a small amount of dental tartar, the trichomonades are so few that it takes some time to find one. But by placing the inoculated tubes at a temperature of 37°C.<sup>2</sup> and then transferring a small quantity of the culture into new medium every 24 hours for 4 or 5 days in succession, the number of the organisms gradually increases and no difficulty was experienced in finding quantities of agglomerating flagellates as well as free individuals in every field. The organisms group together in clusters of varying size around the masses of bacteria (Fig. 1). At lower temperatures (23–27°C.) the growth of bacteria as well as of protozoa proceeds much more slowly than at higher temperatures, and the transfer may be made every 48 hours.

The method of transfer is simple. As the trichomonas grows at the bottom of the tube with bacterial sediments, it is necessary to suck up this precipitate with a capillary pipette and transfer it to new tubes. In both sucking up and transferring precaution should be taken not to stir the culture too much. In this way strains were carried for over twenty generations, and the loss of the strain was usually due to an accident or negligence in the transfer.

A brief note on the *Entamæba gingivalis* Gross (*Entamæba buccalis* Steinberg), which is almost always encountered in this medium, may be made here. In this medium the entameba can easily be kept alive over 24 hours, and with a little precaution sometimes over 48 hours; that is, by keeping the medium always at about 37°C. and by having a sufficient amount of culture fluid. If the alkaline reaction of the medium is reduced by adding drops of a 1 per cent solution of acetic acid to almost the neutral point indicated by litmus paper,

<sup>2</sup> Lynch could not get cultures at 37°C.

the rapidity of the growth of the trichomonas is somewhat reduced, whereas that of the entamebæ is accelerated. By transferring the sediments into a new, already heated medium once every 24 hours, the amebæ can be kept alive over a week; in one instance they were kept alive as long as 10 days. Judging from the fact that only a small amount of the sediment is carried from one culture tube into two, three, or more new tubes each time, the amebæ remain alive and multiply, though not so strikingly as in the case of the trichomonades.

*Morphological and Biological Properties of the Culture Trichomonas.*

The body of the trichomonas is uniformly pear-shaped; but being highly flexible it can take any shape according to circumstances, especially at the time when it passes through the narrow spaces in the bacterial masses, as observed in a fresh preparation. The protrusion of any definite pseudopodium as drawn by Kuczynski (2) in the case of *Trichomonas augusta* has not been seen in our culture, but a prolongation of the protoplasm near the posterior end (Fig. 2) was often observed. This is often used as an anchor, while the main part has a lively oscillating movement (von Prowazek's *haftpseudopodialer Fortsatz*). This prolongation of the protoplasm, however, has nothing to do with a real pseudopodium as we understand it in the case of the ameba, and there is no evidence that it is used for obtaining food. On the other hand, the presence of a cytostome (peristome) is probable, though we could not discern it clearly. In one instance a long chain of bacilli was gradually taken in through a point near the beginning of the flagella. After it became embedded in the protoplasm the chain broke into two parts, one of which was soon cast off from the body through the same point by which it had first entered, while the other seemed to be digested.

The size of the culture trichomonas varies within wide ranges, but it usually measures 10 to 15  $\mu$  in length and 4 to 8  $\mu$  in width (Fig. 11). Sometimes particularly large forms are encountered, about 25  $\mu$  long and 12  $\mu$  wide (Figs. 12 and 13). Flagella at the anterior end are uniformly four in number, radiating out from a basal granule situated just in front of the nucleus. They are unequal in length, in most instances

two of them being a little longer than the other two. The length of the flagella averages 14 to 16  $\mu$ . An undulating membrane rising from a blepharoplast, which lies close to, but differs from the basal granules, is distinctly seen (Fig. 8). The distal end of the limiting line of the membrane usually lies within the body, while its terminal end sometimes projects beyond the margin as a short, free flagellum (Fig. 3).

The axostyle usually seems to take its origin at the distal end of the spindle-shaped nucleus, but sometimes its course may be traced through the nucleus as far up as the basal granule, or very close to it. In its distal course the axostyle runs downward through the body and projects beyond the margin at the posterior end of the organism, forming a free tail 5  $\mu$  or less in length. The nucleus is single, oval, more or less elongated in shape, has a distinct karyosome, and measures 1 to 1.5  $\mu$  by 2 to 3  $\mu$ .

The protoplasm shows no differentiation of ectoplasm and endoplasm, and no contractile vacuoles or chromidial apparatus could be observed other than fine granulation with scattered, coarse particles. In some specimens the protoplasm is filled with ingested bacteria. In preparations stained with Giemsa's solution two or more characteristic purple rings are found, not unlike the forms described by some authors as a stage of so called cyst formation (Fig. 6).

The common method of reproduction seems to be a binary, longitudinal fission (Figs. 14, 15, 16, 17, and 18); multiple fission (or rather budding) may also occur in the culture. Figs. 2 to 10 show a series of successive stages of division, which is complete within from 30 minutes to 1 hour in the hanging drop. The number of daughter cells of multiple fission is not great (4, 6, 8, etc.); they do not seem to separate from each other at the same time, but usually bud off one after another in succession, so that the general impression differs considerably from the usual mode of schizogony as in other protozoa (Figs. 19 and 20). The division of blepharoplast is of the mitotic type (Fig. 19), while that of the nucleus itself is promitotic (Fig. 21). In cultures no form has been observed which may be interpreted as a cyst.



## DISCUSSION AND SUMMARY.

Trichomonades from the mouth were studied by Steinberg<sup>3</sup> who proposed to group them into three distinct types; namely, *Trichomonas elongata*, *Trichomonas caudata*, and *Trichomonas flagellata*. Doflein (3) regards them as probably identical with *Trichomonas hominis*. Opinions differ as to whether or not *Trichomonas vaginalis* Donné and *Trichomonas hominis* Grassi are the same species. Lynch, for instance, believes that they are the same species, while von Prowazek (4), Bensen (5), and others (6, 7) insist that they are different types. Bensen's view seems to be well supported by the difference alleged to be found between the mode of encystment in the two trichomonades, were it not for the fact that our knowledge about the so called cyst of trichomonades is still obscure. According to Alexeieff (8) many of the so called cysts were evidently blastomeres contained in the cell body of the trichomonas. An autogamy alleged to take place in cysts as described by Bohne and von Prowazek (9) has not been confirmed by Dobell (10). And Wenyon (11) contends that it has never been found possible to produce any development of these cysts outside the body on the warm stage as can be done with the cysts of *Entamoeba coli*. Therefore, it is still premature to take the process of encystment into consideration as far as the classification of trichomonas is concerned. On the other hand, Rodenwaldt (12) seems to think that there are many species of trichomonas in the human intestines, and Wenyon has described a new trichomonas from the human intestines (*Macrostoma mesnili* Wenyon).

Further cultural studies in the morphology and biology of these organisms must be carried out in order to solve these problems.

In the light of modern investigations there are five subgenera to be included under the genus *Trichomonas* Donné. They are as follows:

(1) *Protrichomonas* Alexeieff, with three anterior flagella, without an undulating membrane.

(2) *Trichomastix* Bütschli, with three anterior flagella and a trailing flagellum (*Schleppgeissel*) without an undulating membrane.

<sup>3</sup> Steinberg, quoted by Doflein, F., in *Lehrbuch der Protozoenkunde*, Jena, 3rd edition, 1911, 492.



(3) *Trichomonas* Donn , with three anterior flagella and an undulating membrane.

(4) *Macrostoma* Alexeieff, Amend, Wenyon (11), with three anterior flagella and an undulating membrane wedged in a deep groove (peristome).

(5) *Tetratrichomonas* Parisi (13), with four anterior flagella and an undulating membrane.

As far as our culture trichomonas from the human mouth is concerned, it has been shown that it is not strictly a trichomonas and that it should be classed under the subgenus *Tetratrichomonas*.

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## EXPLANATION OF PLATES.

All the specimens were obtained from cultures of *Tetratrichomonas hominis*.

## PLATE 34.

FIG. 1. Dark-field illumination of a colony of *Tetratrichomonas hominis* in culture.  $\times 1,000$ .

## PLATE 35.

Air-dried, fixed in methyl alcohol, and stained with Giemsa's solution. Drawn with camera lucida, oc. 4, obj.  $\frac{1}{2}$ , oil immersion. Tube 160.

FIG. 2. Typical shape.

FIGS. 3 to 9. Different shapes.

FIG. 10. A phase of binary fission.

## PLATE 36.

Moist, fixed in Schaudinn's sublimate alcohol with a few drops of acetic acid, and stained with Heidenhain's iron and hematoxylin.

FIG. 11. Small type.

FIGS. 12 and 13. Large types.

FIGS. 14 and 15. Preliminary phases of binary fission.

FIG. 16. Beginning of binary, longitudinal fission of the protoplasm.

FIGS. 17 and 18. Final phases.

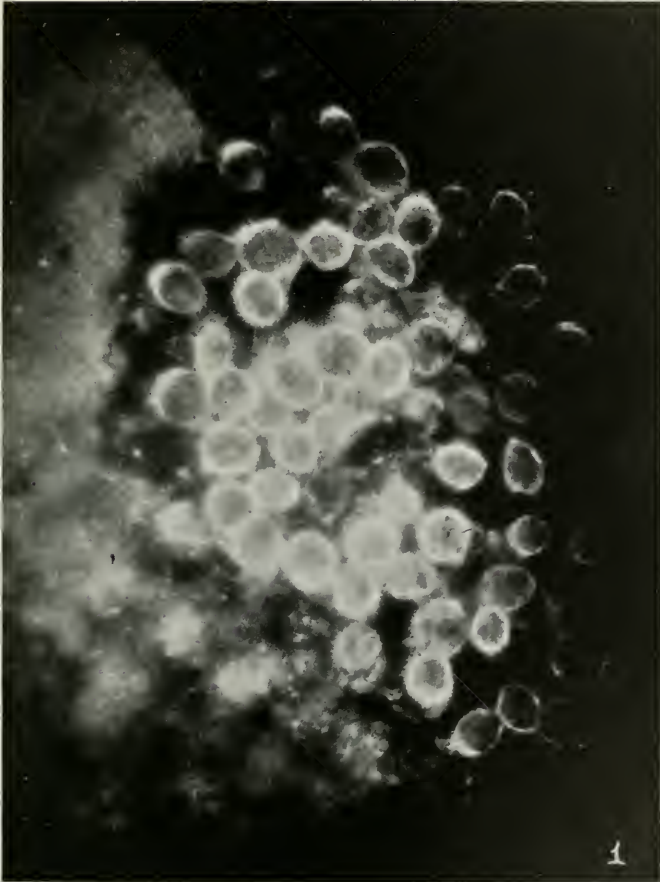
## PLATE 37.

FIG. 19. A phase of multiple fission, showing six daughter nuclei and a mitotic spindle of a basal granule.

FIG. 20. Final stage.

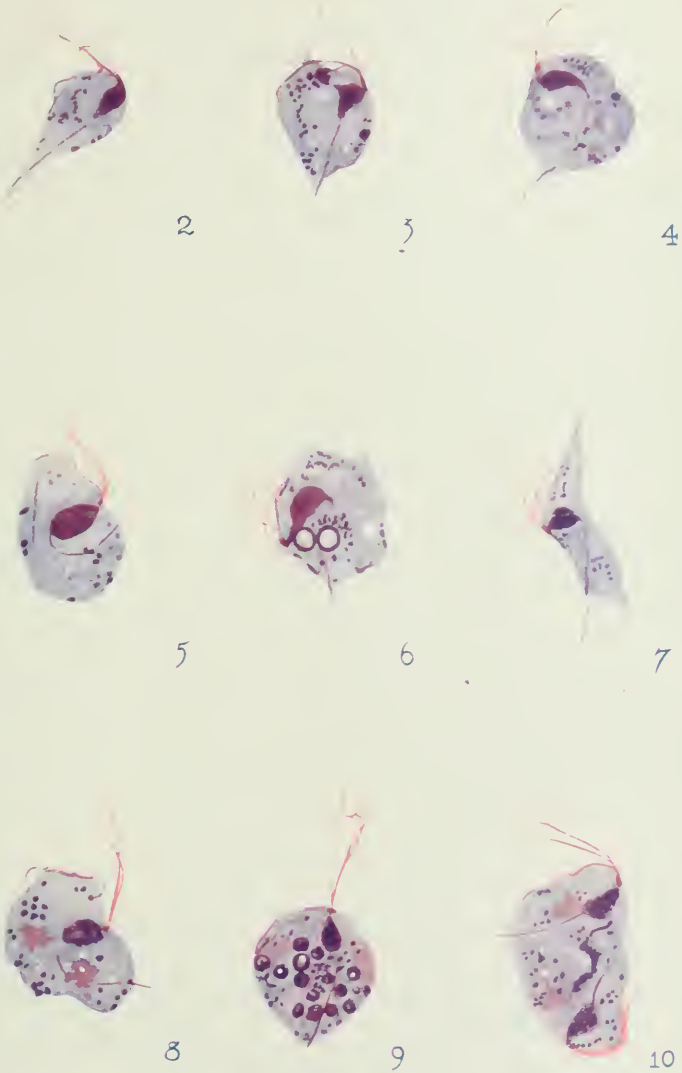
FIG. 21. Promitotic division of the nucleus.





(Obira and Noguchi: Trichomonas of the Human Mouth.)



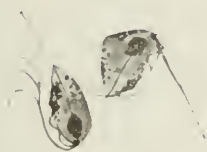


Tokuzo Ohira fec.

(Ohira and Noguchi: Trichomonas of the Human Mouth.)







11



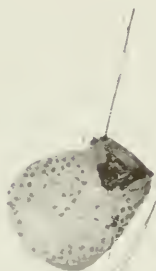
12



13



14



15



16



17



18





19



20



21



## THE DRUG-FASTNESS OF SPIROCHETES TO ARSENIC, MERCURIAL, AND IODIDE COMPOUNDS IN VITRO.

BY SEINAI AKATSU, M.D., AND HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 22, 1916.)

It has been known for some time that in trypanosomiasis the trypanosomes which have survived the first effect of an arsenic germicide, such as atoxyl or arsacetin, offer a greater resistance to a subsequent dose of the same drug. By subjecting the organisms to repeated injections of an arsenic medicament, one tends to create, both in animals and in man, a strain or race which resists the arsenic chemotherapy to such an extent that the term arsenic-fastness has come into existence as a brief designation of the modified strain.<sup>1</sup> A similar phenomenon has been observed in spirochetosis in fowls and mammals. Even with a typical bacterium, there seems to exist a possibility of raising the original resistance against a certain arsenic compound to a considerable degree, as was shown by Marks<sup>2</sup> in his experiment with the paratyphoid bacillus, which he was finally able to cultivate in a medium containing eight times the amount of arsenious acid which the organism was able to withstand at the beginning of his experiment. Marks accomplished this within a period of 3 years by successive transplantations from a weaker to a gradually stronger concentration of the acid.

In syphilis, even before the discovery of *Treponema pallidum*, it had long been suspected that the causative agent of this disease acquires a gradual tolerance to the action of mercurial and iodide preparations. The intermittent form of treatment generally adopted by clinicians, with gradually ascending doses of the medicaments for each course, bears sufficient evidence of this assumption. That

<sup>1</sup> Ehrlich, P., and Hata, S., *Die experimentelle Chemotherapie der Spirillosen*, Berlin, 1910.

<sup>2</sup> Marks, L. H., *Ueber einen arsenfesten Bakterienstamm*, *Z. Immunitätsforsch., Orig.*, 1910, vi, 293.



unicellular organisms, under certain environmental influences, gradually acquire tolerance to certain toxic substances is not inconceivable, inasmuch as instances are not wanting where a group of cells, or an entire organization of much higher multicellular organisms, becomes adjusted to new surroundings when the change is brought about by degrees. In short, the state of drug-fastness is well known among the metazoan organisms.

To establish definitely whether *Treponema pallidum* acquires an increased resistance to various antisyphilitic therapeutic agents is of great practical importance, for the regulation of dosage of medicaments must necessarily be guided by the changes which occur at the same time in the resistance of the parasites. It may be recalled that Ehrlich assumed from analogy with trypanosomiasis and fowl spirochetosis that *Treponema pallidum* becomes readily fast or insensitive to the action of arsenic compounds, and with this idea he evolved his *therapia sterilisans magna*, that is, a total sterilization of *Treponema pallidum* in the system of the infected individual by a single administration of salvarsan, in which he combined a maximum parasitotropic and a minimum organotropic activity. The benefit derived from his efforts to achieve the aim of *therapia sterilisans magna* has been great, but in its practical application salvarsan has fulfilled only a part of Ehrlich's expectation. The consensus of opinion is that salvarsan has to be used in many successive doses, like other antisyphilitic medicaments, in order to obtain the full benefit of the drug. The introduction of salvarsan and neosalvarsan as routine medicaments in the treatment of syphilis raises once more the question whether or not the repeated injections of these preparations tend to produce an arsenic-fast strain of *Treponema pallidum*. This vital point, as well as those with regard to the alleged fastness of the *pallidum* against mercurial and iodide compounds, has never been proved experimentally, probably because of the fastidious character of *Treponema pallidum* in its behavior in the experimental animals available for studying these points.

For ascertaining experimentally that the syphilitic organism becomes gradually more tolerant to the sterilizing effect of various arsenic, mercurial, and iodide preparations, four different methods suggest themselves: (1) to determine *in vivo* the resistance of *Trep-*

*onema pallidum* to the germicidal action of the drug in question, both before and after the administration of the drug; (2) to subject *in vitro* the *pallidum* derived from normal and treated animals to the action of various concentrations of the drug and then to inoculate them into a series of suitable animals; (3) to cultivate the organism instead of inoculating it into animals as in (2); (4) to determine the degrees of tolerance of pure cultures of *Treponema pallidum* to a given medicament by testing the organisms *in vitro* against gradually increasing doses of the drug.

The first two methods are practically unavailable for the present problem, since there is no animal in which an accurate estimation of the relation between the number of the parasites and the severity and character of the lesions produced could be made with uniformity, and no method is yet known which will permit the cultivation of the *pallidum* directly from the animal tissue emulsions, as would be required if we were to adopt the third procedure. We have, therefore, left these three methods out of consideration and followed the fourth, the only procedure which may be carried out with the various factors under ready control and with a fair degree of constancy in results.

In the present work, we have chosen not only the syphilitic spirochete, but also some allied non-pathogenic varieties, and tested them against some of the representatives of antisyphilitic medicaments. We do not consider that the phenomena observed *in vitro* on pure cultures of spirochetes can be directly transferred to the processes occurring in the animal or human body, but we believe that the observations reported in this paper warrant sufficient interest as such.<sup>3</sup>

#### EXPERIMENTAL.

##### *Method.*

As the test objects, three strains of pure cultures of *Treponema pallidum*, one of *Treponema microdentium*, and one of *Spirochæta refringens* were employed. Against each of the above organisms, salvarsan, neosalvarsan, bichloride of mercury, and Lugol's solution

<sup>3</sup> Akatsu, S., The Resistance of Spirochetes to the Action of Hexamethylene-tetramine Derivatives and Mercurial and Arsenic Compounds, *J. Exp. Med.*, 1917, xxv, 363.

were used. The solutions of salvarsan and neosalvarsan in sterile distilled water were each time freshly prepared, the proportion being 1 : 10,000. The solution of bichloride of mercury was made up to 1 : 10,000 during the early part of the experiment and became 1 : 1,000 as the concentration was gradually increased in order to keep the volume of the solution of the sublimate to be added to the media within a certain limit. Lugol's solution was prepared by dissolving 1 gm. of iodine and 2 gm. of potassium iodide in 300 cc. of distilled water. In the experiment a 1 : 10 dilution was first used, then an undiluted solution, (1 part of iodine, 2 parts of potassium iodide, and 300 parts of distilled water), and finally one three times as concentrated as the usual undiluted solution (1 part of iodine, 2 parts of potassium iodide, and 100 parts of distilled water).

Because of certain technical difficulties, the use of solid media was restricted to a small series. The majority of the experiments were carried out by means of a fluid medium consisting of equal parts of neutral bouillon and ascitic fluid with a piece of fresh kidney of a normal rabbit. To maintain the relative volumes of the drug solutions and the culture media, none of the former was added in a volume greater than 1 cc. or less than 0.05 cc. When the requisite amount of the drug solution exceeded 1 cc., a corresponding quantity of a tenfold stronger concentration was employed in order to keep the volume within the specified limit. The total volume of the culture medium in each tube was made uniformly 5 cc., including the quantity of drug solution added.

A series of test-tubes was set up, each containing a piece of fresh rabbit kidney, and inoculated with the pure culture of the spirochete. To the tubes were added first the ascitic bouillon (equal parts), and then the drug solution in the quantity indicated for each tube. The whole content of the tube was thoroughly mixed by gently inverting the tube a few times, then covered with a layer of sterile paraffin oil, and finally incubated at 36°C. in an anaerobic apparatus. At the end of 14 days, the cultures were examined for growth under the dark-field microscope. From the tubes showing varying degrees of growth, transfers were made into a new series of media containing different doses of the drug. Again, after a fortnight's incubation, transfers were similarly made, the process being repeated every 2 weeks.

*Results of Experiments.*

It was possible to find certain quantities of each drug, the addition of which to the culture media completely inhibited the growth of the organisms. As the quantities of the drug were gradually reduced, the organisms grew in corresponding abundance. With a very small quantity of the drug, no appreciable effect upon the growth was seen. But between the two extremes there were doses which distinctly showed the inhibitory influence of the drug as indicated by the retardation and meagerness of growth. In the border-line tubes the organisms were fewer in number, less active, and often granular in appearance. Numerous degenerated forms were seen in these tubes.

To conform with the purpose of the present work, transfers were made both from the border-line tubes and from those containing smaller amounts of the drug into a number of new media, to some of which ascending doses of the drug solution had been added. After 14 days in the incubator at 36°C., the cultures were examined for growth in the manner already described. From repeated observations it was discovered that no growth or very scant growth was obtainable when a transfer was made from one of the border-line tubes to a tube containing an equal quantity of the drug, and that even if there was any growth in the second transfer, it was not further transferable. The organisms seemed to succumb to the continued effect of the drug in such a concentration. Under these conditions there was no evidence that the organisms had in any way acquired an increased resistance to the drug.

On the other hand, the organisms growing fairly well in the tubes with somewhat smaller amounts of the drug were found capable of transplantation into new media, in the presence of not only the same amount, but also of somewhat larger doses. In other words, the minimal inhibitory doses became gradually larger with each transfer. In the accompanying tables we present a general aspect of the effect of drug-containing media upon the gradual development of tolerance by the spirochetes.

The point of interest here lies in the fact that the organisms under the influence of moderate quantities of an adverse drug acquire a greater resistance to it and are capable of doing so progressively up to a certain point. As will be described, the various organisms em-

ployed in this experiment behaved differently towards different agents, and our results permit the tentative generalization that the group of treponemata gains, under certain conditions, a definite, though rather slight degree of resistance to salvarsan and neosalvarsan, while its resistance to the bichloride of mercury (and iodide?) preparation is strikingly augmented under similar circumstances.

In Tables I and II the reaction of different spirochetes towards the influence of each of the chemical preparations used in the present work is shown.

TABLE I.

First transplantation from drug media to drug media.		Transferred to the tubes containing.				
		Dose.				No drug.
		1	2	3	4	
From tubes containing the doses and showing the growth indicated.	Dose 1, causing total inhibition (—).	—	—	—	—	—
	Dose 2, causing slight growth (<+).	—	—	—	—	+
	Dose 3, causing moderate growth (<+).	—	<+	+	+	+
	Dose 4, causing good growth (+).	—	+	+	+	++
	Dose 5, causing no inhibition (++)	—	<+	<+	+	++
	Control tube without any drug, showing luxuriant growth (++)	—	<<+	<+	+	++

TABLE II.

Third transplantation from drug media to drug media.		Transferred to the tubes containing.				
		Dose.				No drug.
		1	2	3	4	
From the second transplants containing the doses and showing the growth indicated.	Dose 2, showing moderate growth (<+).	<+	+	++	++	++
	Dose 3, showing good growth (+).	<+	+	++	++	++
	Dose 4, showing good growth (+).	<+	+	++	+	++
	Control tube without any drug, showing luxuriant growth (++)	—	<<+	<+	+	++



Table III represents the results of experiments with salvarsan and shows how various *pallidum* strains, and *microdentium* and *refringens*, withstood the action of the drug.

TABLE III.

*Salvarsan.*

*Maximum Doses in Which Abundant Growth Still Occurred on Successive Transplantations.*

	Generation in drug media.						
	1	2	3	4	5	6	7
<i>T. pallidum.</i>	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Strain McD.....	0.03	0.05	0.06	0.07	0.07	0.08	0.1
“ R.....	0.02	0.03	0.04	0.04	0.05	0.1	0.12
“ Z. A.....	0.02	0.04	0.04	0.05	0.08	0.12	Accident
<i>T. microdentium</i> .....	0.01	0.02	0.04	0.06	0.07	0.07	“
<i>S. refringens</i> .....	0.02	0.02	0.04	Accident.	0.04	0.04	0.05

The figures denote the amount in milligrams of salvarsan contained in 5 cc. of the culture medium. The first column shows the doses of salvarsan in which various strains of the spirochetes still thrived; the second those in which the organisms grew well on the first transplantation; the third those for the second transplantation, etc. It will be seen that the initial tolerance of the *pallidum* and *refringens* was slightly greater than that of the *microdentium*, while the rate with which tolerance increased, up to the end of the fifth and sixth transplantation, was greatest with the *microdentium* and *pallidum*, these having attained about six to seven times their original tolerance within  $3\frac{1}{2}$  months. The *refringens* still remained sensitive to the drug during this time.

Table IV shows the results with neosalvarsan, and they agree in general with those obtained with salvarsan, except that larger quantities were tolerated on account of its weaker action. The *pallidum* and *microdentium* reached four to five times their initial tolerance, while the tolerance of *refringens* was least affected.

In general, the tolerance of the *pallidum* and *microdentium* to salvarsan and neosalvarsan was definitely raised, while that of the *refringens* seems to have been least increased. With the *pallidum*



TABLE IV.

*Neosalvarsan.**Maximum Doses in Which Abundant Growth Still Occurred on Successive Transplantations.*

	Generation in drug media.						
	1	2	3	4	5	6	7
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
<i>T. pallidum.</i>							
Strain McD.....	0.03	0.07	0.08	0.1	0.12	0.15	0.15
“ R.....	0.02	0.05	0.06	0.06	0.07	0.08	0.08
“ Z. A.....	0.03	0.04	0.06	0.06	0.07	0.08	0.1
<i>T. microdentium</i> .....	0.02	?	0.03	0.07	0.1	0.1	0.1
<i>S. refringens</i> .....	0.03	0.06	0.08	0.08	0.1	0.1	0.1

strains, the rise in tolerance was gradual and the probable limit was not reached until the fifth or sixth transfer, which was made at the end of the 12th or 14th week. With the *microdentium* and *refringens*, especially the latter, the limit of tolerance was reached much sooner, and it could not be raised further after the fourth transfer. In salvarsanized media, the *refringens* reached the limit on the second transfer and remained unchanged for the rest of the experimental period.

The results obtained with bichloride of mercury, compared with those with salvarsan and neosalvarsan were striking. Table V shows that the initial tolerance of all the spirochetes was almost the same, being 0.01 mg. per 5 cc. of the medium. One of the *pallidum* strains tolerated 0.02 mg. The increase in tolerance rapidly rose from the first transfer and progressed gradually for the following generations. In *Treponema pallidum* the limit was not reached until the 16th week (seventh transfer), while that of the *microdentium* had already been reached on the 8th week (third transfer), and that of the *refringens* on the 12th week (fifth transfer). Even the *pallidum* strains could not tolerate much higher concentrations, the absolute limit being 1 mg. in 5 cc. of the medium, when the color of the tissue, as well as of the ascitic fluid, becomes gray within a few days at 36° C., and black streaks appear in the tissue.

The striking feature of the experiment is the high rate of increase in tolerance through the organisms' becoming accustomed to the

TABLE V.

*Bichloride of Mercury.**Maximum Doses in Which Abundant Growth Still Occurred on Successive Transplantations.*

	Generation in drug media.							
	1	2	3	4	5	6	7	8
<i>T. pallidum.</i>	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Strain McD.....	0.02	0.07	0.13	0.17	0.2	0.23	0.5	0.7
“ R.....	0.01	0.03	0.07	0.1	0.2	0.3	0.7	
“ Z. A.....	0.01	0.02	0.07	0.07	0.15	0.3	0.5	0.5
<i>T. microdentium</i> .....	0.01	0.02	0.07	0.1	0.1	0.1	0.1	0.1
<i>S. refringens</i> .....	0.01	0.015	0.03	0.07	0.1	0.3	0.3	0.3

mercurial salt. With the *pallidum*, it was between 35 and 70 times the initial tolerance; with the *refringens*, about 30 times; and with the *microdentium*, 10 times. We did not believe that these delicate organisms could be made resistant to the action of so simple and powerful an inorganic disinfectant as bichloride of mercury. The above observations seem to point to the possibility that bichloride of mercury forms, with certain constituents (proteins and lipoids) of the culture medium, a compound which induces tolerance on the part of the spirochetes.

In Table VI the results obtained with Lugol's solution are given. The figures in the first and second columns do not indicate the maximum doses in which the spirochetes could grow, but those which

TABLE VI.

*Lugol's Solution.**Amounts Contained in Successive Transplantations.*

	Generation in drug media.					
	1	2	3	4	5	6
<i>T. pallidum.</i>	cc.	cc.	cc.	cc.	cc.	cc.
Strain McD.....	0.03	0.05	0.5	1.5	2.5	2.5
“ R.....	0.03	0.04	0.5	0.7	1.5	2.5
“ Z. A.....	0.02	0.05	0.5	1.0	2.0	2.0
<i>T. microdentium</i> .....	0.01	0.06	0.3	0.8	0.8	2.0
<i>S. refringens</i> .....	0.02	0.03	0.3	0.5	1.0	2.0

had been arbitrarily added to the media at the beginning of the experiments, with the purpose of gradually accustoming the organisms to the action of iodine and iodide. The doses given in the third to the sixth columns indicate the quantities added to the media where the spirochetes could still grow well. It may be stated here that these organisms, before passing through the iodinated media for some generations, could not grow in the presence of 0.7 cc. of Lugol's solution in 5 cc. of the culture media.

The color of Lugol's solution soon disappears from the media when the mixture is placed at 36°C. Even the addition of 2.5 cc. did not preserve the brown color over 48 hours. The most important factor affecting the antispirochetal action of the iodine solution was found to be the size of the fresh tissue added. The larger the tissue, the safer the spirochetes were from the inhibitory or sterilizing influence of the preparation. For this reason our experiments were carried out with as uniform a size of the tissue as practicable for each tube, and the results presented in Table VI may be considered approximately correct. It is true also with salvarsan, neosalvarsan, and bichloride of mercury, but the interference from this source was much less disturbing than with the iodine solution. Thus our figures in all the experiments should be taken as expressing merely the approximate values.

At the end of the experiments, a comparative study of the drug-fast and the ordinary stock cultures of the spirochetes in relation to their resistance to salvarsan, neosalvarsan, bichloride of mercury, and Lugol's solution was undertaken. The results are shown in Tables VII, VIII, IX, and X.

Attempts to carry out parallel experiments in solid media met with certain technical difficulties, in that the drugs had to be added while the mixture of the ascitic fluid and agar was still in fluid condition; that is, at a temperature of about 45°C. When salvarsan, neosalvarsan, and bichloride of mercury were mixed with the media, a distinct turbidity resulted, and solidification of the media at once followed, sometimes rendering a uniform distribution of the drug impossible. It was for this reason probably that we were unable to obtain results of definite constancy to warrant the assumption of the existence of an increased drug tolerance in the spirochetes when

TABLE VII.

*Salvarsan.*

In 5 cc. of media containing.	Strain McD., which grew in the tube containing 0.06 mg.	Strain McD., never in contact with salvarsan.
	Growth.	Growth.
<i>mg.</i>		
0.2	—	—
0.1	<+	—
0.05	+	—
0.01	++	+
0.005	++	++
No drug.	++	++

TABLE VIII.

*Neosalvarsan.*

In 5 cc. of media containing.	Strain R., which grew in the tube containing 0.1 mg.	Strain R., never in contact with neosalvarsan.
	Growth.	Growth.
<i>mg.</i>		
0.2	—	—
0.1	<+	—
0.05	<+	—
0.01	+	<+
0.005	++	<+
No drug.	++	++

TABLE IX.

*Bichloride of Mercury.*

In 5 cc. of media containing.	Strain Z. A., which grew in the tube containing 0.5 mg.	Strain Z. A., from original solid culture, never in mercuric chloride media.
	Growth.	Growth.
<i>mg.</i>		
1.0	—	—
0.5	<+	—
0.1	+	—
0.05	++	—
0.01	++	<+
No drug.	++	++

TABLE X.  
*Lugol's Solution.*

In 5 cc. of media containing.	Strain McD., which grew in the tube containing 2 cc.	Strain McD., never in contact with Lugol's solution or iodide.
	Growth.	Growth.
cc.		
2.0	<+	—
1.0	+	—
0.5	++	±
0.1	++	+
0.05	++	+
No drug.	++	++

passed through the drug-containing media for several generations. Nor was it possible to demonstrate the acquired drug tolerance by means of solid media, which was easily seen in the fluid media. We are not prepared to offer an explanation for this phenomenon. Perhaps in fluid media the drugs enter into combination with certain tissue ingredients of the media and in this form induce a gradual tolerance on the part of the spirochetes, whereas with solid media the drugs are held in the agar in such a manner as not to become modified enough to produce increased resistance in the spirochetes.

With regard to the duration of the acquired drug tolerance of the spirochetes, a series of experiments was carried out in which the drugged strains were returned to the ordinary fluid media without any drug. From each successive generation, tolerance tests were made with varying concentrations. The results are summarized in Table XI.

As may be seen from Table XI, the acquired tolerance to various drugs decreases fairly rapidly. Within a period of 8 weeks, during which four transfers through ordinary media were made, the acquired tolerance of the *pallidum* and *refringens* to salvarsan and neosalvarsan entirely disappeared, and that of *microdentium* disappeared even sooner. The disappearance of the acquired tolerance to bichloride of mercury was more gradual, requiring about 10 weeks for complete restoration of the normal sensitiveness to this salt. In this respect, the paratyphoid strain used by Marks<sup>2</sup> differs greatly from the spirochetes, as the former passed through forty-six passages before returning to the initial tolerance.

TABLE XI.

Maximum Doses of Each Drug in Which Various Drug-Fast Strains of the Spirochetes Still Grew Well after Being Returned to Drug-Free Media for Successive Generations.

	Salvarsan.			Neosalvarsan.			Bichloride of mercury.		
	<i>T. pallidum</i> .*	<i>T. microdentium</i> .	<i>S. refringens</i> .	<i>T. pallidum</i> .*	<i>T. microdentium</i> .	<i>S. refringens</i> .	<i>T. pallidum</i> .*	<i>T. microdentium</i> .	<i>S. refringens</i> .
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
After one generation in drug-free media....	0.1	0.05	0.05	0.15	0.1	0.1	0.5	0.1	0.1
“ two generations in “ “ ....	0.05	0.03	0.03	0.1	0.05	0.08	0.3	0.1	0.1
“ three “ “ “ “ ....	0.05	0.02	0.02	0.05	0.02	0.04	0.05	0.07	0.05
“ four “ “ “ “ ....	0.03	0.01	0.02	0.03	0.02	0.03	0.04	0.04	0.03
“ five “ “ “ “ ....	0.02	0.01	0.02	0.03	0.02	0.03	0.02	0.01	0.01
“ six “ “ “ “ ....	0.02	0.01	0.02	0.03	0.02	0.03	0.02	0.01	0.01

\* *Pallidum*, Strain McD.

## SUMMARY.

In the foregoing experiments we attempted to determine whether or not, by subjecting several varieties of spirochetes to increasing doses of certain chemotherapeutic agents, a gradual increase of resistance to the latter could be shown. For this purpose, pure cultures of *Treponema pallidum*, *Treponema microdentium*, and *Spirochæta refringens* were used against the action of salvarsan, neosalvarsan, bichloride of mercury, and iodine-iodide potassium solution *in vitro*. For culture media, the usual ascites-broth-tissue medium as well as solid ascites-agar-tissue medium was used. After permitting the spirochetes to grow for a fortnight in media containing certain quantities of each drug, transfers were made from tubes showing various degrees of growth to the next series of tubes containing the same drug in still higher concentrations, and similar transfers repeated every 2 weeks. The results of the experiments may be briefly summarized as follows:

1. *Treponema pallidum* and *Treponema microdentium* have, within 3 to 4 months, increased their tolerance to salvarsan and neosalvarsan to five and one-half times their original mark. With *Spirochæta refringens* the increase was about three times.



2. Against the action of bichloride of mercury, the amount of increased tolerance of *Treponema pallidum* was about 35 to 70 times the original, while that of *Treponema microdentium* was about 10 times as much and was reached within 10 weeks. *Spirochæta refringens* resisted 30 times the original dose.

3. There was an unmistakable increase of resistance of these spirochetes to the action of the iodine-iodide solution (Lugol's solution) when they were grown for several generations in fluid media containing the iodine solution, but the rate of increase between the initial and the acquired tolerance was slight. In general, the addition of Lugol's solution to fluid media has a weak inhibitory influence upon the growth of the spirochetes, requiring for the total suppression of growth a quantity of over 0.7 cc. to 5 cc. of the culture media. The tolerance reached was for about three times that amount.

4. A similar tolerance phenomenon has not been established when employing a solid instead of a fluid medium containing the drugs. No explanation is offered except a suggestion that the drugs held in the agar do not enter into combination with certain tissue constituents of the medium as they are able to do with tissue elements in fluid media. This may be a factor necessary for inducing drug tolerance in these organisms *in vitro*.

5. The increased drug-fastness *in vitro* has a limit beyond which no further advance can be made. This limit varies with different species of spirochetes.

6. The acquired drug-fastness *in vitro* gradually disappears when the spirochetes are cultivated again in the drug-free media for several generations.

# THE RESISTANCE OF SPIROCHETES TO THE ACTION OF HEXAMETHYLENETETRAMINE DERIVATIVES AND MERCURIAL AND ARSENIC COMPOUNDS.

By SEINAI AKATSU, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The effect of the most common physical and chemical agents upon pure cultures of different spirochetes has already been studied by Bronfenbrenner and Noguchi.<sup>1</sup> In the present series of experiments we studied the action of a large number of hexamethylenetetramine derivatives and new mercurial and arsenic compounds upon several varieties of spirochetes in pure cultures. The bactericidal strengths of the drugs upon different bacilli or cocci have already been tested,<sup>2-5</sup> and it seemed to us to be of sufficient interest to examine also their germicidal powers for spirochetes, since spirochetes and bacteria often show a marked difference with regard to their resistance to a given chemical substance.

<sup>1</sup> Bronfenbrenner, J., and Noguchi, H., On the Resistance of Various Spirochaetes in Cultures to the Action of Chemical and Physical Agents, *J. Pharm. and Exp. Therap.*, 1912-13, iv, 333.

<sup>2</sup> Jacobs, W. A., and Heidelberg, M., The Quarternary Salts of Hexamethylenetetramine. I-VIII, *J. Biol. Chem.*, 1915, xx, 659, 685; xxi, 103, 145, 403, 439, 455, 465; Mercury Derivatives of Aromatic Amines. I. Contribution to the Structure of Primary and Secondary *p*-Aminophenylmercuric Compounds, xx, 513.

<sup>3</sup> Jacobs, W. A., The Bactericidal Properties of the Quarternary Salts of Hexamethylenetetramine. I. The Problem of the Chemotherapy of Experimental Bacterial Infections, *J. Exp. Med.*, 1916, xxiii, 563.

<sup>4</sup> Jacobs, W. A., Heidelberg, M., and Amoss, H. L., The Bactericidal Properties of the Quarternary Salts of Hexamethylenetetramine. II. The Relation between Constitution and Bactericidal Action in the Substituted Benzylhexamethylenetetraminium Salts, *J. Exp. Med.*, 1916, xxiii, 569.

<sup>5</sup> Jacobs, W. A., Heidelberg, M., and Bull, C. G., The Bactericidal Properties of the Quarternary Salts of Hexamethylenetetramine. III. The Relation between Constitution and Bactericidal Action in the Quarternary Salts Obtained from Halogenacetyl Compounds, *J. Exp. Med.*, 1916, xxiii, 577.

## EXPERIMENTAL.

The new compounds employed for the present work<sup>6</sup> are given in Table I. Most of these compounds are readily soluble in distilled water. A few exceptions are marked with an asterisk or a dagger in the table. The insoluble preparations were first ground in a mortar with a small quantity of water and then 0.1 N hydrochloric acid (to those marked with an asterisk) or 0.1 N sodium hydroxide (to those marked with a dagger) was added, until the drug passed into a clear solution. After that more water was added until a 1 per cent solution was obtained. Most of these water-insoluble drugs required from 2 to 4 cc. of a 0.1 N solution of hydrochloric acid or sodium hydroxide to dissolve 0.1 gm. of the substance.

To determine the intensity of the action of these chemical compounds, the following procedure was adopted. A number of dilutions in descending strengths were made with each compound, such as 0.01, 0.001, 0.0001, etc., using distilled water as a diluent. From each of the dilutions 1 cc. was measured into each of the small test-tubes in series, and to the latter three drops of a stock culture of the spirochetes were added.

The stock culture used in the first series of experiments consisted of fluid cultures of *Treponema pallidum* as grown by Noguchi's method<sup>7</sup> and contained a considerable number of actively motile organisms, as seen under the dark-field microscope.

The contents of each tube having been thoroughly mixed by shaking, the tubes were left in a water bath and kept at 37°C. for 1 hour. At the end of that time, transplants into new media were made with the contents of the tubes, in order to determine in what dilution each of the chemical compounds was able to sterilize the spirochetes completely. A microscopic examination alone is insufficient to determine whether the organisms are killed or not. For the purpose of setting up new cultures, Noguchi's solid medium containing

<sup>6</sup> I wish to acknowledge the courtesy of Dr. W. Jacobs and Dr. M. Heidelberger, of The Rockefeller Institute for Medical Research, who kindly placed these chemical compounds at my disposal.

<sup>7</sup> Noguchi, H., A Method for Cultivating *Treponema pallidum* in Fluid Media, *J. Exp. Med.*, 1912, xvi, 211.

TABLE I.

Preparation No.	
9	<i>p</i> -Bromobenzylhexamethylenetetraminium chloride.
16	<i>o</i> -Xylylenedi-hexamethylenetetraminium "
19	2-Nitro-3, 4-dimethoxybenzylhexamethylenetetraminium chloride.
21	2-Oxy-3-carbomethoxynaphthobenzylhexamethylenetetraminium chloride.
28	5-Chloromethylvanillin + hexamethylenetetramine.
29	5-Chloromethylsalicylic acid + "
40	<i>p</i> -Iodobenzylhexamethylenetetraminium bromide.
46	<i>o</i> -Nitrobenzylhexamethylenetetraminium chloride.
47	<i>p</i> -Nitrobenzylhexamethylenetetraminium "
50	Methylhexamethylenetetraminium iodide.
84	Chloroacetamide + hexamethylenetetramine.
86	Oxymethylchloroacetamide + "
90a	Ethyl bromoacetate + "
96	Chloroacetylaniline + "
97	$\beta$ -Acetoxy- $\alpha$ -chloroacetylnaphthobenzylamine + hexamethylenetetramine.
102	Chloroacetyl- $\alpha$ -naphthylamine + hexamethylenetetramine.
107	Chloroacetylbenzylamine + hexamethylenetetramine.
109	Chloroacetyl- $\beta$ -naphthylamine + "
111	<i>o</i> -Methylchloroacetylbenzylamine + "
112	<i>p</i> -Chloroacetylaminobenzoic ethyl ester + hexamethylenetetramine.
114	Chloroacetylurea + hexamethylenetetramine.
121	Phenoxyethylhexamethylenetetraminium bromide.
122	<i>p</i> -Bromochloroacetylaniline + hexamethylenetetramine.
126	Chloroacetylaminoozotoluene + "
134	Chloroacetyl- <i>p</i> -anisidine + "
138	Chloroacetylacetylphenylhydrazine + hexamethylenetetramine.
142	Chloroacetethylamide + hexamethylenetetramine.
146	Menthyl bromoacetate + "
147	Bromoethylphthalimide + "
148	<i>p</i> -Nitrobenzoic bromoethyl ester + hexamethylenetetramine.
150	Bromoethyl benzoate + hexamethylenetetramine.
158	$\beta$ -Iodopropionyl- <i>o</i> -anisidine + "
163	<i>p</i> -Ethoxyphenyl bromoethyl ketone + hexamethylenetetramine.
164	Chloroacetyl- $\psi$ -cumidine + hexamethylenetetramine.
168	<i>p</i> -Acetamino- $\omega$ -bromoacetophenone + "
171	<i>m</i> -Chloroacetylaminomethyl benzamide + "
172	<i>m</i> -Chloroacetyl- $\alpha$ , $\alpha$ -phenylbenzylhydrazine + hexamethylenetetramine.
174	<i>m</i> -Chloroacetylaminooethyl anisate + hexamethylenetetramine.
204	3- $\omega$ -Bromoacetylquinaldine + "
218	Tribromo- <i>p</i> -cresyl bromoethyl ether + "
219	<i>p</i> -Chloroacetylaminoleucomalachite green + " *
229	<i>p</i> -Chloroacetylaminobenzeneazo- <i>p</i> '-dimethylaniline + hexamethylenetetramine.
232	<i>p</i> -Chloroacetylaminobenzeneazo- <i>p</i> '-diethylaniline + "

TABLE I—*Concluded.*

Preparation No.	
234	$\alpha$ -Naphthyl bromoethyl ether + hexamethylenetetramine.
239	<i>o</i> -Acetaminophenyl bromoethyl ether + “
242	<i>p</i> -Chloroacetylaminodiethylaniline + “
244	Chloroacetyl aminoethyl <i>p</i> -nitrobenzoate + “
249	<i>p</i> -Chloroacetylaminodipropylaniline + “ *
252	<i>p</i> -Chloroacetylaminotetraethyl- <i>p'</i> , <i>p''</i> -diaminotriphenylmethane + hexamethylenetetramine.
253	Chloroacetyl diethylamine + hexamethylenetetramine.
255	<i>p</i> -Cyanobenzylhexamethylenetetraminium chloride.
257	<i>o</i> -Chloroacetylaminophenyl benzoate + hexamethylenetetramine.
261	Chloroacetyltriphenylmethylamine + “
262	Chloroacetyl leucoauramine + “ *
263	Chloroacetyl aminoethyl <i>o</i> -nitrobenzoate + “
267	Chloroacetyl aminoethyl $\beta$ -naphthoate + “
271	Chloroacetyl-N-phenyl aminoethyl <i>p</i> -nitrobenzoate + hexamethylenetetramine.
272	<i>m</i> -Acetamino- <i>p</i> -tolyl $\omega$ -iodoethyl ketone + “
273	Chloroacetyl ethyl aminoethyl <i>p</i> -nitrobenzoate + “
278	$\alpha$ , $\beta$ -Diphenylchloroacetyl aminoethanol + “
280	<i>m</i> -Chloroacetyl aminoacetophenone + “
282	$\alpha$ -Phenyl- $\alpha$ -oxy- $\beta$ -chloroacetyl aminoethane + hexamethylenetetramine.
283	<i>p</i> -Nitrobenzoyl aminoisopropyl chloroacetate + “
286	Iodopropanol + hexamethylenetetramine.
289	2-Chloroacetyl amino-3-oxy-3-methylbutane + hexamethylenetetramine.
291	Chloroacetyl- <i>o</i> -methylphenoxyethylamine + “
293	$\beta$ -Chloroacetyl amino- $\gamma$ -butanol + “
298	$\beta$ -Phenyl- $\beta$ -oxy- $\gamma$ -chloroacetyl aminopropane + “
301	$\beta$ -Naphthyl bromoethyl ether + hexamethylenetetramine.
303	2-Oxy-3, 5-dibromobenzyl bromide + “ † †
308	Chloroacetyl- <i>m</i> -iodoaniline + “
309	Chloroacetyl-5-iodo- <i>o</i> -toluidine + “
M1	4-[ <i>p</i> -Oxybenzeneazo]-phenylmercuric acetate.
M4	[ <i>o</i> -Oxybenzylideneamino]-phenylmercuric “ †
M7	1-Amino-2-[ <i>p</i> -naphthaleneazophenylmercuric acetate]-5-sulfonic acid.

\* Grind up in a mortar with a little water and add 0.1 N hydrochloric acid until dissolved.

† Treat as above, using 0.1 N sodium hydroxide instead of hydrochloric acid.

‡ Difficultly soluble.

a piece of fresh sterile rabbit kidney and ascitic agar (17 cc. in each tube) was employed. The quantity of transplanted fluid containing the spirochetes and the drug was approximately 0.2 cc. for each



tube. Thus, a certain amount of the drug was necessarily carried over into a new culture together with the spirochetes, which were suspended in different solutions of the drug. After 14 days' incubation at 37°C., the results of the culture were ascertained by both macroscopic and dark-field examinations.

In the preliminary series of experiments it was found that different drugs varied considerably with regard to their spirocheticidal power. Thus, some had the power to kill the organism in a dilution of 1:100, but not in 1:1,000, while others were effective in 1:1,000 and not in 1:10,000, etc. Each of the drugs was again subdivided for the purpose of determining more accurately its disinfecting power against the spirochetes. For example, in the case of a drug which was found effective in 1:100 and not in 1:1,000, five subdivided dilutions between both extremes, such as 1:100, 1:250, 1:500, 1:750, 1:1,000, were tested.

In this series the manner of testing the spirocheticidal powers was exactly the same as in the first series. After 14 days the results were taken with the aid of the dark-field microscope (Table II). The color of each preparation, as it appears in a fresh 1 per cent solution, is noted in the table.

TABLE II.

Preparation No.	Appearance of a 1 per cent fresh solution.	Result.	
		Concentration sufficient to kill spirochetes.	Concentration in which spirochetes survived.
9	Clear.	1 : 1,000	1 : 2,500*
16	Brown.	1 : 2,500	1 : 5,000
19	Opaque.	1 : 2,500	1 : 5,000
21	Slightly greenish opaque with sediment.	1 : 2,500	1 : 5,000
28	Brown.	1 : 750	1 : 1,000
29	Clear.	1 : 2,500	1 : 5,000
40	Slightly opaque.	1 : 750	1 : 1,000
46	Opaque.	1 : 250	1 : 500
47	Clear.	1 : 750	1 : 1,000
50	"	1 : 100	1 : 250
84	"	1 : 1,000	1 : 2,500
86	"	1 : 250	1 : 500

\* Grind up in a mortar with a little water and add 0.1 N sodium hydroxide until dissolved.



TABLE II—*Continued.*

Preparation No.	Appearance of a 1 per cent fresh solution.	Result.	
		Concentration sufficient to kill spirochetes.	Concentration in which spirochetes survived.
90a	Clear.	1 : 1,000	1 : 2,500
96	Slightly opaque.	1 : 1,000	1 : 2,500
97	“ yellowish opaque.	1 : 1,000	1 : 2,500
102	Opaque.	1 : 500	1 : 750
107	Clear.	1 : 500	1 : 750
109	Milky opaque.	1 : 1,000	1 : 2,500
111	Slightly “	1 : 2,500	1 : 5,000
112	Opaque.	1 : 1,000	1 : 2,500
114	Clear.	1 : 1,000	1 : 2,500
121	“	1 : 250	1 : 500
122	Opaque.	1 : 2,500	1 : 5,000
126	Reddish yellow.	1 : 250	1 : 500
134	Slightly opaque.	1 : 2,500	1 : 5,000
138	Clear.	1 : 750	1 : 1,000
142	“	1 : 1,000	1 : 2,500
146	Slightly opaque.	1 : 750	1 : 1,000
147	Clear.	1 : 1,000	1 : 2,500
148	Slightly opaque.	1 : 250	1 : 500
150	Clear.	1 : 500	1 : 750
158	Milky opaque.	1 : 1,000	1 : 5,000
163	“ “	1 : 500	1 : 750
164	“ “	1 : 2,500	1 : 5,000
168	Clear.	1 : 750	1 : 1,000
171	“ slight sediment.	1 : 1,000	1 : 2,500
172	Milky opaque.	1 : 2,500	1 : 5,000
174	Opaque.	1 : 500	1 : 750
204	Slightly reddish yellow.	1 : 2,500	1 : 5,000
218	Milky opaque.	1 : 2,500	1 : 5,000
219	Green.	1 : 5,000	1 : 7,500
229	Yellowish red.	1 : 500	1 : 750
232	Reddish yellow.	1 : 1,000	1 : 2,500
234	Clear.	1 : 500	1 : 750
239	“	1 : 1,000	1 : 2,500
242	Yellowish opaque.	1 : 1,000	1 : 2,500
244	Opaque.	1 : 2,500	1 : 5,000
249	Milky.	1 : 500	1 : 750
252	Green.	1 : 1,000	1 : 2,500
253	Clear.	1 : 1,000	1 : 2,500
255	“	1 : 1,000	1 : 2,500
257	Yellowish opaque, a little sediment.	1 : 1,000	1 : 2,500
261	Milky.	1 : 1,000	1 : 2,500

TABLE II—*Concluded.*

Preparation No.	Appearance of a 1 per cent fresh solution.	Result.	
		Concentration sufficient to kill spirochetes.	Concentration in which spirochetes survived.
262	Slightly opaque.	1 : 1,000	1 : 2,500
263	“ yellowish opaque.	1 : 1,000	1 : 2,500
267	Milky.	1 : 2,500	1 : 5,000
271	Brown.	1 : 1,000	1 : 2,500
272	Milky with sediment.	1 : 5,000	1 : 7,500
273	Slightly opaque.	1 : 1,000	1 : 2,500
278	“ with sediment.	1 : 250	1 : 500
280	Clear.	1 : 1,000	1 : 2,500
282	“	1 : 1,000	1 : 2,500
283	Opaque.	1 : 500	1 : 750
286	Clear.	1 : 500	1 : 750
289	“	1 : 2,500	1 : 5,000
291	Opaque.	1 : 1,000	1 : 2,500
293	“	1 : 250	1 : 500
298	Clear.	1 : 1,000	1 : 2,500
301	Opaque.	1 : 1,000	1 : 2,500
303	Brown with slight sediment.	1 : 1,000	1 : 2,500*
308	Milky.	1 : 750	1 : 1,000
309	“	1 : 750	1 : 1,000
Atoxyl.	Clear.	1 : 50	1 : 100
M1	Yellow.	1 : 50,000	1 : 75,000
M4	Light yellow.	1 : 50,000	1 : 75,000
M7	Dark red.	1 : 25,000	1 : 50,000

A parallel series of experiments was conducted with a number of the most common disinfectants and antisymphilitic medicaments, in order to compare the action of new chemical compounds with that of standard ones. The results obtained with these substances are given in Table III.

These experiments were made with a strain belonging to a medium type of *Treponema pallidum*, as has been stated. For the comparison, two more strains, belonging one to a thin and the other to a thick type of the *pallidum*, as well as several other species of spirochetes (*refringens*, *microdentium*, *mucosum*, *calligyrum*, etc.) were tested with some of the chemicals. For this purpose M1, one of the powerful compounds against the *pallidum*, and No. 253, a much weaker drug, were chosen. The results are given in Table IV.

TABLE III.

Name of drug.	Appearance of a 1 per cent fresh solution.	Result.	
		Concentration sufficient to kill spirochetes.	Concentration in which spirochetes survived.
Phenol.	Clear.	1 : 2,500	1 : 5,000
Formalin.	"	1 : 750	1 : 1,000
Lysol.	Slightly opaque.	1 : 5,000	1 : 7,500
Bichloride of mercury.	Clear.	1 : 100,000	1 : 500,000
Salvarsan.	Yellow.	1 : 7,500	1 : 10,000
Neosalvarsan.	"	1 : 2,500	1 : 5,000
Sodium iodide.	Clear.	1 : 10	1 : 25
Potassium iodide.	"	1 : 10	1 : 25
Lugol's solution.	Deep reddish brown.	1 : 75	1 : 100
Iodoxybenzoic acid.	Clear.	1 : 500	1 : 1,000
Trypозofrol.	Dark red.	1 : 25,000	1 : 50,000
Neotrypозofrol.	Deep "	1 : 250	1 : 1,000
Sodium cholate.	" brown.	1 : 5,000	1 : 7,500
" glycocholate.	" "	1 : 2,500	1 : 5,000
" taurocholate.	Light "	1 : 2,500	1 : 5,000
" oleate.	Yellowish opaque.	1 : 7,000	1 : 10,000
Saponin.	" transparent.	1 : 7,500	1 : 10,000
Cholesterol.	Insoluble.	No action.	No action.
Cobra lecithide.	Yellowish opaque.	1 : 1,000	1 : 5,000
" venom.	" "	1 : 1,000	1 : 5,000

TABLE IV.

Spirochete.	M1			No. 253	
	1:10,000	1:25,000	1:50,000	1:1,000	1:2,500
<i>T. pallidum</i> , large type.....	1:10,000	1:25,000	1:50,000	1:1,000	1:2,500
" " small ".....	—	+	—	—	+
" <i>calligyrum</i> .....	—	—	+	—	+
" <i>mucosum</i> .....	—	—	+	—	+
" <i>microdentium</i> .....	—	—	+	—	+
<i>S. refringens</i> .....	—	—	+	—	+

Different species of spirochetes seem to have about the same resistance to the action of these drugs.

In order to determine the relative resistance of spirochetes on the one hand, and certain bacteria on the other, *Bacillus dysenteriae* Shiga and *Streptococcus* were subjected to the action of these compounds in a parallel series and under similar experimental con-

ditions. For this purpose, a 24 hour bouillon culture of the dysentery bacilli and an agar slant culture of streptococci were employed. The results are recorded in Table V.

TABLE V.

Preparation No.	<i>B. dysenteriae.</i>				<i>Streptococcus.</i>		
	1:100	1:1,000	1:10,000	1:100,000	1:100	1:1,000	1:10,000
9	—	+					
16	+						
21	+						
29	+						
40	—	+			—	+	
46		—	+		—	+	
50	—	+			+		
84	—	+			—	—	+
86		—	+				
90a	—	+			—	+	
107	—	+			—	+	
114					—	+	
142	—	+			—	+	
146	—	+			—	+	
150	—	+			+		
171	—	+					
218	—	+					
232	—	+			—	+	
239	—	+					
242	+				—	+	
244	—	+			—	+	
253	—	+			—	+	
257		—	+				
278	—	+					
283	+				—	+	
286	—	+					
298	—	—	+				
Atoxyl.	+						
Lugol's solution.	—	+					
Neosalvarsan.	—	+		+			
Bichloride of mercury.	—	—	—	—			

## DISCUSSION AND SUMMARY.

The majority of the 76 new compounds possessed spirocheticidal powers ranging between 1:1,000 (30) to 1:2,500 (14), while only 2 attained the power of 1:5,000, 1 of 1:25,000, and 2 of 1:50,000. On the other hand, 8 killed the spirochetes in a dilution of 1:750, 10 in 1:500, 7 in 1:250, 1 in 1:100, and 1 in 1:50.

It may be mentioned that the 2 (M1, M4) of 1:50,000 and 1 (M7) of 1:25,000 belong to the mercury compounds, and that mercuric chloride kills the organisms in a dilution of 1:100,000 under the same experimental conditions. It is also interesting to compare some of the more common chemicals and therapeutic reagents. Phenol is spirocheticidal in a dilution of 1:2,500, lysol in 1:5,000, formalin in 1:750, salvarsan in 1:7,500, and neosalvarsan in 1:2,500. Thus, of the new compounds there are at least 14 which have the same spirocheticidal power *in vitro* as has neosalvarsan.

It is of interest to note that nine compounds possessing the 1:1,000 spirocheticidal power showed only one-tenth of the antiseptic action when tested upon *Bacillus dysenteriae* and *Streptococcus*, while some showed an even greater difference in this respect. Nos. 16, 21, 29, 218, and 244 were effective in a dilution of 1:2,500 for spirochetes and in a 1:100 or lower dilution for the bacteria just referred to. Preparations 46 and 84 appear to exert about the same effect both on the spirochetes and the bacteria, neither being very strong. Atoxyl killed the spirochetes in a 1:50 dilution.

One of the most striking results was obtained with various hemolytic substances. Neufeld and von Prowazek<sup>8</sup> found that spirochetes, unlike bacteria in general, are highly susceptible to the lytic action of sodium taurocholate and saponin, and they considered that this phenomenon was of differential diagnostic value in determining plant and animal organisms. Their observations were confirmed by Gonder,<sup>9</sup> who found, however, that spirochetes, especially treponemata,

<sup>8</sup> Neufeld, F., and von Prowazek, S., Ueber die Immunitätserscheinungen bei der Spirochaetenseptikämie der Hühner und über die Frage der Zugehörigkeit der Spirochaeten zu den Protozoen, *Arb. k. Gsndtsamte.*, 1907, xxv, 494.

<sup>9</sup> Gonder, R., Spironemacea (Spirochaeten), in von Prowazek, S., *Handbuch der pathogenen Protozoen*, Leipzig, 6 Liefg., 1914, 671.

offer a great deal of resistance at the beginning, but finally undergo lysis, with their cell bodies swollen up or macerated. In the present experiment we have found that these substances not only bring about cytolysis of the spirochetes in higher concentrations, but also kill them without causing a gross destruction of the cells in very high dilutions; thus, sodium cholate in 1:5,000, sodium glycocholate and taurocholate in 1:2,500 each, saponin in 1:7,500. Sodium oleate, one of the most powerful hemolytic agents<sup>10, 11</sup> killed the spirochetes in 1:7,000, and cobra lecithide and native cobra venom in a dilution of 1:1,000 each.

Neither sodium nor potassium iodide displayed any marked spirocheticidal property (1:10 only), while iodine in the form of Lugol's solution was able to kill them at 1:75.

In conclusion I wish to acknowledge my indebtedness to Dr. Hideyo Noguchi for his assistance and advice.

<sup>10</sup> Madsen, T., and Noguchi, H., Toxines et antitoxines. Saponine-cholestérine, *Overs. k. Danske Vidensk. Selsk. Forh.*, 1904, 457.

<sup>11</sup> Noguchi, On Certain Thermostabile Venom Activators, *J. Exp. Med.*, 1906, viii, 87.





# THE INFLUENCE OF CARBOHYDRATES ON THE CULTIVATION OF SPIROCHETES.

By SEINAI AKATSU, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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While a newly isolated bacterium would usually, as a matter of bionomic routine, be tested soon for its power of fermentation of various carbohydrates, nothing systematic has been done in this direction with the group of organisms classed as spirochetes. The lack of investigation along this line may be partly due to the technical difficulties which still surround the cultivation of these organisms, and probably also to the limited number of workers in the field.

The present paper is a report of experiments which deal with the influence of starch and sugars upon pure cultures of various spirochetes. Attention has been directed to the fermentation phenomena as well as to the effect upon the growth and morphology of the organisms, since we are mindful of the fact that the addition of certain of these substances to some bacterial cultures may bring about almost incredible involution of forms<sup>1</sup> or sometimes induce spore formation in a bacterium which is otherwise not sporiferous.<sup>1,2,3</sup>

## EXPERIMENTAL.

### *Method.*

For the present work, the following substances were tested: amygdalin, arabinose, beerwort, dextrin, galactose, glycogen, glucose, inulin, lactose, levulose, maltose, mannite, raffinose, saccharose, and

<sup>1</sup> For example, Noguchi noticed that the colon bacillus undergoes striking morphological changes when cultivated in beerwort, producing gigantic pleomorphic forms of various shapes. Some of the large specimens measured about 30  $\mu$  in length and 2 or 3  $\mu$  in width in the swollen portion.

<sup>2</sup> Noguchi, H., Sporulation of the Group of *Bacillus aerogenes capsulatus*, *Proc. N. Y. Path. Soc.*, 1907, vii, 196.

<sup>3</sup> Fitzgerald, M. P., The Induction of Sporulation in the Bacilli Belonging to the *Aerogenes capsulatus* Group, *J. Path. and Bacteriol.*, 1911, xv, 147.

starch. These fifteen carbohydrates were added to the tubes containing the usual medium (equal parts of ascitic fluid and bouillon with a piece of fresh tissue, a total volume of 10 cc. to each tube) in a proportion of 1 : 100. Inoculations were then made into the media with each of the seven strains of *Treponema pallidum* and one each of *Treponema calligyrum*, *Treponema microdentium*, *Treponema mucosum*, and *Spirochæta refringens*. The culture tubes were covered with a layer of sterile paraffin oil and placed in an incubator at 36°C. Examinations of the spirochetes were made from time to time, for a period of 3 months with the first generation and of 2 months with the second generation.

### *Results of Experiments.*

It was soon found that none of the spirochetes employed for the present study produced gas in the presence of these carbohydrates, and even after many weeks there was no turbidity or precipitate in any of the cultures which would indicate that the acid produced, if any, was sufficient in amount to cause coagulation of the proteins of the media. No difference in appearance could be discerned between the cultures in the sugar media and those in the sugar-free media, save the slight opalescence in the cultures of the *microdentium* in the glycogen and glucose media. When examined under the dark-field microscope, all the spirochetes were found to have grown vigorously in all the sugar media except those containing amygdalin, glycogen, glucose, and lactose. In the glycogen medium, the *microdentium* grew for several days, but quickly degenerated, though it multiplied abundantly in the beerwort medium. Glucose medium induced good growth of all the spirochetes in the first generation, but *microdentium* and some *pallidum* strains failed to grow when transferred to a new medium containing the same sugar. The effects of glycogen and glucose are similar. *Treponema mucosum*, which is similar to *Treponema microdentium* in morphology and odor-producing property, seems to be distinguished from the latter by its indifference to the addition of glucose to the medium. In Table I the results of the dark-field examination of the cultures are given as recorded after 3 weeks for the first generation and 4 weeks for the second.

The morphological features of the spirochetes were not appreciably influenced, except that the terminal appendages<sup>4</sup> were much more in evidence in the specimens grown in a sugar medium than in the sugar-free control cultures. In the culture of *microdentium*, the spirochetes showed numerous refractile spherical bodies, laterally attached, similar to those first described by Noguchi in the specimens of the *pallidum* cultures. Since the life of *Treponema microdentium* in sugar media is shorter than in a sugar-free medium, that is, since it grows earlier and degenerates earlier, the observed phenomenon may have an intimate connection with a process of degeneration such as plasmoptysis, where the minute granules do not represent a resistant form (spore) of the spirochetes, for they succumb to the degeneration as do the spirochetes themselves.

As may be seen from Table I, the addition of glycogen and glucose had a decidedly unfavorable effect upon the cultures of *Treponema calligyrum*, *Treponema microdentium*, *Treponema mucosum*, and some strains of the *pallidum*. *Treponema mucosum* grew most luxuriantly in all media except in that containing glycogen, and its uniform length, regular curves, and energetic motility indicate that this organism finds an ideal medium when these substances are added.

The reactions of the cultures varied from almost neutral to distinctly acid when tested with litmus paper. The acidity was strongest in the *microdentium* culture in glycogen and glucose media, and somewhat weaker in the media containing galactose, lactose, maltose, and inulin. Only a faint acidity was found in any of the cultures of spirochetes in sugar media except in glycogen and glucose media, where some of them produced a distinct acidity. In the control cultures without any carbohydrate, the reaction of the *microdentium* only was faintly acid, all the rest being practically neutral. By titrating the total acidity of 10 cc. of the fluid culture, it was found that the highest acidity as represented in the glycogen and glucose cultures of the *microdentium* was 0.1 N 4.8 cc., while in the cultures showing a weaker acidity, it was between 0.1 N 2 cc. to 0.1 N 3.2 cc. The ciphers for the control cultures varied from 0.1 N 0.8 cc. to 0.1 N 2 cc. Leaving a more exact qualitative and quantitative determination

<sup>4</sup> Noguchi, A Method for the Pure Cultivation of Pathogenic *Treponema pallidum* (*Spirocheta pallida*), *J. Exp. Med.*, 1911, xiv, 99.



In the table the sign — indicates absence of spirochetes; +, less than 100; ++, a number between 100 and 200; +++, more than 200; and +++, innumerable spirochetes per field under Leitz oc. 3,  $\frac{1}{2}$  oil immersion. The letter *c* denotes the presence of long threads or chains of spirochetes, while *g* indicates that there were numerous granular particles due to the degenerated spirochetes.

for another occasion, we believe that the fact has been established that some sugars are attacked to a certain extent by some of the spirochetes employed for the present experiment, and especially by *Treponema microdentium*. Of course, it is impossible from the present experiment to determine how much of the acidity found should be ascribed to the split products of the sugars. While the amount of acidity in the glycogen or glucose media did not produce coagulation of the proteins, nevertheless the early disintegration of the organisms, particularly in the case of *Treponema microdentium* shows the effect of the changes associated with the acid production in the media.

#### SUMMARY.

Various carbohydrates have been added to the fluid cultures of different strains of spirochetes in order to determine the behavior of the latter toward the carbohydrates. In the present experiment, amygdalin, arabinose, beerwort, dextrin, galactose, glycogen, glucose, inulin, lactose, levulose, maltose, mannite, raffinose, saccharose, and starch were tested with seven strains of *Treponema pallidum* and one strain each of *Treponema calligyrum*, *Treponema microdentium*, *Treponema mucosum*, and *Spirochæta refringens*. The results may be summarized as follows:

1. In the media containing glycogen and glucose, *Treponema microdentium* did not grow as vigorously as in other sugar media, and an earlier degeneration set in. One strain of the *pallidum* and the *calligyrum* and *mucosum* showed a poor growth in the glycogen medium. Similarly, there was little growth in the second transfer of these spirochetes in the glucose medium. The growth of the spirochetes in the media containing carbohydrates other than those just mentioned was generally good, and no difference could be distinguished between these and the control cultures without any carbohydrate. The only phenomenon which might be interpreted as indicating



a favorable influence of these media upon growth was the abundant growth of the *mucosum*, which showed uniform length, regular curves, and active motility somewhat better than in the sugar-free medium.

2. The height of acidity was found in the cultures containing glycogen and glucose in the *microdentium*, amounting to 0.1 N 4.8 cc. for 10 cc. of the fluid culture. In the other sugar media the acidity varied between 0.1 N 2 cc. and 0.1 N 3.2 cc. for the same amount. In the control cultures, the acidity fluctuated from 0.1 N 0.8 cc. to 0.1 N 2 cc. There was no visible alteration in the appearance of the media after the spirochetes had grown for 3 or 4 weeks. In the case of *Treponema microdentium*, a slight opalescence developed in the glycogen and glucose media after several weeks' standing, but there was no precipitation or coagulation of the proteins of the culture media.

3. There was no unusual morphological change in the spirochetes grown in the media containing any of the carbohydrates employed. The only phenomena which should be mentioned are (a) the frequent presence of the terminal appendages (or projections) in the *refringens* and in most of the *pallidum* strains, and (b) the appearance of minute, refractile spherical bodies along the side of the spirochetes in the *microdentium* cultivated in the glucose or glycogen media. Judging from the earlier degeneration of the species in the above mentioned media, these peculiar bodies may be interpreted as indicating a phase of plasmoptysis associated with the unfavorable surroundings prior to degeneration. Experimental evidence was not found for considering these spherules as a resistant or spore form of the spirochete.

In conclusion I wish to express my indebtedness to Dr. Hideyo Noguchi for his assistance and advice.

## THE ABSORPTION OF FAT IN PARTIALLY, AND IN COMPLETELY DEPANCREATIZED DOGS

By CHARLES W. McCLURE, M.D., BETH VINCENT, M.D., AND  
JOSEPH H. PRATT, M.D.

*(From the Laboratory of Medicine, Harvard Medical School, Boston.)*

(Received for publication, December 8, 1916.)

For more than 50 years physiologists have studied the relation of the pancreas to the absorption of fat from the intestine, but the problem still remains unsolved. Many studies have been made on animals to determine whether the pancreatic juice is essential to the absorption of fat, or whether fat absorption is regulated by an internal secretion of the pancreas.

In 1856 Claude Bernard (1) presented detailed evidence for his claim that the pancreatic juice was of great importance in the process of digestion and in the absorption of fat by the intestines. In two dogs out of a series of ten, he found, after injecting melted suet or other substances into the main pancreatic duct, that the stools became very fatty. From the 11th to the 15th day one of these dogs passed large amounts of clear fat. In the 3rd week the feces contained less fat, and when the animals were autopsied it was found that the pancreatic juice was again discharging into the intestine.

The importance of the experiments of Claude Bernard was not appreciated for many years. The physiologists of his time, unable to confirm his observations, opposed the view that the pancreatic secretion was essential to the absorption of fat. Bernard replied to his opponents and showed that their experiments were without value, as they failed to exclude all the pancreatic secretion from the intestine. They remained unconvinced, and their conclusion, as expressed by Colin (2), that the pancreatic juice is not necessary for the digestion and absorption of the normal amount of fat, is the one held today by most investigators.

In 1890 Abelman (3), working under Minkowski, studied fat absorption in dogs after partial and complete depancreatization. All communication between the pancreatic remnant and intestines was destroyed in the partially depancreatized dogs. These animals absorbed from 25 to 65.8 per cent of fats derived from olive oil, horse flesh, or butter. After the complete removal of the pancreas, the dogs were unable to absorb any of these fats, unless raw pancreas was fed, but they did absorb 28 to 90 per cent of the fat of milk.

The unabsorbed fats, however, were found to be split in the intestines. 35 to 85 per cent of the fat in fresh stools consisted of fatty acids and soaps. The fat in the small intestines contained 30 per cent of fatty acids and that in the colon 76 per cent, 12 hours after feeding neutral fat to a completely depancreatized dog.

The observations that fats were split but not absorbed, unless raw pancreas was added to the food, led Abelman to conclude that: All fats, with the exception of milk fat, unquestionably require pancreatic juice to be absorbed. He explained fat absorption in the partially depancreatized dog by assuming that the agent effecting absorption is carried to the lumen of the intestine in some way as yet unknown.

Sandmeyer (4) reported finding in the stools of a partially depancreatized dog a greater amount of fat than had been fed. The pancreatic remnant in this dog consisted of a portion of the processus lienalis 3 cm. in length. A persistent glycosuria developed 1 month after the operation. Consecutive metabolism experiments were made throughout a period of 5 months. The percentages of fat lost in the dog's stools varied from 9.1 to 102 per cent in twenty-six experiments; from 104.6 to 111.2 per cent in eight; and from 122.08 to 163.5 per cent in four.

Rosenberg (5) separated the pancreas from the duodenum in a dog with the object of shutting out all the pancreatic secretion from the intestine. For a time the absorption of fat remained normal, but within 2 months a slight reduction occurred. In a series of fifteen experiments the absorption of fat averaged about 85 per cent and only once fell as low as 64 per cent.

In 1906 Lombroso (6), working with dogs, found that a large percentage of fat was absorbed, after all direct communication between the pancreas and duodenum was supposed to have been removed. This was also true, if the pancreas was extirpated except for a small portion which discharged its juice through a permanent fistula in the abdominal wall. When complete depancreatization had been effected, there was either slight or no absorption of fat. He concluded from his findings that the absorption of fat from the intestine is governed by an internal secretion of the pancreas.

Niemann (7) observed good absorption after tying the ducts. In five experiments on one dog 91.2 to 98.7 per cent of the fat in the food was absorbed; in three experiments on a second dog, 85.6 to 98.5 per cent.

Burkhardt (8) concluded from experiments on one dog that fat absorption was not regulated by an internal secretion of the pancreas. He extirpated all of the pancreas except the processus uncinatus. This portion was transplanted under the skin and a cutaneous pancreatic fistula was produced.

In the first metabolism experiment the animal was allowed to lick the fistula and in this way obtained pancreatic secretion. 80 per cent, or 47 gm., of the fat ingested was absorbed. In a second experiment in which licking the fistula was prevented, only 13 per cent, or 8 gm., of the fat was taken up by the intestine.

The findings in this pair of experiments were not verified in two succeeding

pairs of experiments. In these, no appreciable difference occurred in the fat absorption whether the dog did or did not lick the fistula.

Fleckseider (9) made a similar study. The splenic portion and the upper part of the body of the pancreas were extirpated. The remainder of the gland was separated from the duodenum and the main duct and surrounding pancreatic tissue transplanted into the abdominal wall, so that the secretion discharged externally. In only one experiment was there poor absorption (20 per cent) of fat, and later the same animal absorbed a much larger percentage (65 per cent), although it was prevented from obtaining any pancreatic juice. In one metabolism experiment a dog was allowed to lick the fistula, and less fat was absorbed than in the experiments when all pancreatic secretion was withheld. He concluded that the power of absorption of fat by the intestinal mucous membrane is chiefly dependent on an internal secretion of the pancreas.

Lombroso (10) in his later investigation repeated Burkhardt's work and obtained contradictory results. Sometimes more fat was absorbed when the dog was free to lick the pancreatic secretion from the permanent fistula and sometimes less. The absorption of fat in the animals with a subcutaneous graft varied from 48.5 to 80.3 per cent.

Pratt, Lamson, and Marks (11) in experiments on a series of five dogs found that when the pancreas was entirely separated from the duodenum, serious disturbance of fat absorption took place. The percentage of fat taken up by the intestine ranged from 4.8 to 76.6.

Jansen (12) partially extirpated the pancreas of a dog and transplanted the remainder under the skin. In three experiments the animal absorbed 74.9 to 76.4 per cent of the fat ingested. After the removal of the pancreatic graft the fat lost in the feces ranged from 15 to 156 per cent.

Visentini (13) devised a method of determining at autopsy whether or not pancreatic juice had been secreted into the intestine. In five dogs in which he had succeeded in permanently excluding all pancreatic secretion after ligature and resection of the ducts, the fat absorbed ranged from 28.7 to 44.0 per cent. In four dogs in which the corpus pancreatis and processus uncinatus were extirpated, the smallest percentage of fat absorbed was 8.7, and the largest was 25.7.

Much of the experimental work summarized above is open to criticism. There are three chief sources of error.

1. Failure to exclude all the pancreatic juice from the intestine. The difficulty of excluding permanently the pancreatic juice was shown clearly by Claude Bernard. Hess (14) thought that this was chiefly due to the presence of accessory ducts which were left untied. He showed that dogs might have three or four pancreatic ducts. This anomaly is probably much less common than Hess believed. Visentini failed to find more than two in a series of twenty dogs.

In 1909, Pratt, Lamson, and Marks showed that after the pancreatic ducts were tied and cut between double ligatures a sinus quickly formed between the main duct and the lumen of the intestine. Visentini in a careful investigation has demonstrated that after tying and resecting the two excretory ducts the flow of pancreatic juice may frequently be reestablished by the formation of a sinus.

2. Absorption experiments of too short duration. Abelmann's experiments lasted only 1 day and Burkhardt's 2 days. Some of Jansen's experiments were of only 1 and 2 days' duration. It is now generally recognized that trustworthy results cannot be obtained in such a short time.

3. No intervening period between experiments. Lombroso in his earlier studies often began a second experiment the same day that the preceding one was finished. We have found that dogs may pass stools containing the residue from a single meal for a period of 2 to 3 days. Therefore, in spite of the use of carmine to demarcate the stools, the possibility of the collection, during the second experiment, of feces which belonged to the first cannot be denied. In the final results this error would produce too high a figure for fat absorption in the first experiment, while the figure for fat absorption in the second experiment would be too low. This source of error can account for the results obtained by Burkhardt in his first two metabolism experiments, an explanation supported by the fact that in the two experiments the percentages of fat in the feces were nearly the same (23.8 and 26.3 per cent), but the amount of dried stool was nearly four times as great in the second as in the first.

In the animals studied by Pratt, Lamson, and Marks, rapid and progressive atrophy of the pancreas occurred. Pratt and Spooner (15) found that the power to assimilate glucose was rapidly reduced after tying the ducts, a fact which indicated damage to the internal function of the pancreas. Hence it might be asserted that the experiments of Pratt, Lamson, and Marks in which they found poor fat absorption after separating the pancreas from the duodenum did not disprove Lombroso's theory that the absorption of fat is largely due to some internal function of the pancreas. It should be pointed out, however, that Pratt and his coworkers found that the disturbance in fat absorption developed as soon as pancreatic secre-



tion was absent from the intestine while it was several weeks before there was a marked drop in the power to assimilate glucose.

Lombroso and Fleckseder published accounts of experiments which showed that the fat was fairly well absorbed when a subcutaneous pancreatic graft was made which was allowed to secrete externally, although the remainder of the gland had been extirpated. If the ducts secrete freely, then the degeneration of the gland tissue from back pressure does not occur. According to the view held by Pratt, Lamson, and Marks, it would make no difference in the absorption of fat whether the gland underwent degeneration or remained intact, provided the pancreatic juice did not enter the intestine.

It is a difficult procedure to remove all the pancreatic tissue from the wall of the duodenum, but to explain Lombroso's and Fleckseder's results by ascribing them to technical errors on their part would be unfair until their experiments had been repeated and refuted.

After complete extirpation of the pancreas it is held that little, if any fat is absorbed, except milk fat. This conclusion is based on the experiments of Abelman and Lombroso. If true, it furnishes strong support for the theory that the presence of pancreatic tissue in the body, even when not connected with the intestine, aids in some way the absorption of fat. In all the experiments of Pratt, Lamson, and Marks, in which all pancreatic juice was excluded from the intestine, some absorption of fat took place. In only one experiment was as little as 4.8 per cent absorbed. If no absorption of fat occurs when the pancreas is totally removed, then the experiments published from this laboratory would indicate either that an internal pancreatic function regulated fat absorption, or that the presence of pancreatic tissue in the body in some other way affected the power of the intestinal wall to take up the fat. Abelman, Rosenberg, Pflüger (16), and Burkhardt held that the pancreatic juice, after it is shut out from the duodenum, continues to form, but is taken up by the blood and carried to the other digestive glands and by them secreted into the intestines. Experiments by Lombroso overthrew this theory.

The first part of the present study deals with the absorption of fat in dogs with subcutaneous transplants of the pancreas which discharge their secretion externally. In the second part the results of experiments on completely depancreatized dogs are presented.



*Methods.*

*Partial Pancreatectomy and the Formation of a Cutaneous Pancreatic Fistula.*—Ether anesthesia was used in all the operations. Before etherization a subcutaneous injection of morphine was given. The processus lienalis and corpus pancreatis were completely extirpated, and great care was taken to remove from the duodenal wall every bit of adherent pancreatic tissue. The processus uncinatus of the pancreas was then freed from all its attachments except where the blood vessels entered at the lower end. Leaving these vessels intact, the major portion of the processus uncinatus was transplanted under the skin of the abdominal wall. This was accomplished by first incising the parietal peritoneum about 3 cm. to the right of the median abdominal incision. Then by blunt dissection the muscles were penetrated and a space for the pancreatic remnant was made in the subcutaneous tissue. After incising the skin a hemostat was passed through this space into the abdomen. By means of the hemostat the pancreatic remnant was drawn into the subcutaneous space. The distal end of the remnant was drawn through the incision to a little beyond the level of the skin. It was now fastened by a few fine stitches to the edge of the peritoneum and the skin.

This method was modified in Dog 4. The subcutaneous transplant consisted of the processus uncinatus and that portion of the body of the pancreas containing the main duct. The duct was preserved intact by excising that portion of the duodenal wall which contained the opening of the duct. The blood vessels entering this portion of the intestine were preserved. The portion of the pancreatic remnant containing the duct was made the distal end of the subcutaneous transplant.

*Extirpation of the Subcutaneous Transplant.*—In a second stage of the experiments the pancreatic remnant was completely extirpated. The abdominal portion of the remnant was first removed. This was done through an opening in the abdomen just to the right of the original median incision. Care was taken to close the opening made in the peritoneum. The remainder of the pancreatic transplant was removed through a skin incision.

*Food.*—The food consisted of a mixture of six parts of finely chopped,

boiled horse flesh, three parts of cracker dust, two parts of butter, and four parts of water. The butter was melted in boiling water and then intimately mixed with the solid ingredients. Fresh food was prepared for each feeding.

*Metabolism Experiments on Dogs with a Subcutaneous Pancreatic Graft.*—A period of from 5 to 7 days elapsed after operation before the dogs would take food readily. The experiments were then begun. A meal of milk was given 24 hours previous to the beginning of the experiment. This produced a thin, light colored stool quite different in appearance from the more formed deep yellow or gray stools resulting from the solid food of the metabolism experiment. 20 to 30 gm. of powdered charcoal were intimately mixed with the food of the first meal of each experiment. The resulting stools were very dark gray or black and easily separated from feces derived from other food. Feedings were made once daily over a period of 4 or 5 days. No food was given during the 48 hours succeeding the last meal of an experiment. Then bread and milk mixed with 20 to 30 gm. of charcoal were given. This permitted the collection of all feces belonging to the metabolism experiment. Each animal was kept in the same metabolism cage throughout an experiment except at the time of feeding, when it was transferred to a clean cage.

*Metabolism Experiments on Completely Depancreatized Dogs.*—These dogs received no food for 24 hours prior to the operation for the removal of the subcutaneous pancreatic remnant. Experiments were begun the next day after this operation. With the exception of this preliminary period of starvation the technique of the experiments was the same as described for partially depancreatized dogs. After the completion of one metabolism experiment a period of not less than 3 days elapsed before beginning another experiment.

*Preservation of Specimens for Analysis.*—One-fifth of the amount of food mixture eaten at each meal was preserved for analysis. It was mixed with 95 per cent alcohol containing 1 to 2 per cent by volume of hydrochloric acid and dried to constant weight on a water bath at 50–55°C. After drying, the food was finely triturated in a mortar and preserved in sealed jars. The same procedure was carried out with the stools, except that the entire amount of feces was preserved.

*Analytical Methods.*—Nitrogen was determined by the Kjeldahl method. Specimens were analyzed in duplicate. 0.5 to 1 gm. was used in each determination.

Fat was determined by the Folin-Wentworth (17) method. Specimens were analyzed in duplicate. 2 to 3 gm. of sieved material were used for each analysis. Extraction was carried on for a period of 20 hours. This method was not devised for the determination of the fat content of foodstuffs. However, we found that the same results were obtained by drying the fat, extracted by this method from our food mixture and from milk at 95°C., as when dried to constant weight at 55°C.

*Interpretation of Findings.*—The factors to be considered in comparing the results of absorption experiments are the percentage and weight of the fat absorbed in relation to the amount ingested and the weight of the animal. In the past, workers in this field have drawn their deductions from the percentages of absorption of the fat ingested. This method of expressing results does not tell the whole story regarding the fat absorbed. A relatively high percentage of absorption may represent but a small amount of actual fat, while a lower percentage may represent a great deal more fat. Not only should the percentage of absorption and the actual amount in grams be considered, but also the quantity of fat ingested. Within certain limits the larger the amount fed to an animal, the greater will be the amount absorbed by the animal. Size is an important factor if the number of grams of fat absorbed is to be compared in different dogs. In animals appearing to be about equally nourished the weight offers a simple and fairly reliable means for comparison of the size.

The metabolism in a completely depancreatized dog with the resulting diabetes is greatly disturbed. Animals after total pancreatectomy usually remain in suitable condition for experimentation but a short time. Because of this, it was necessary to work rapidly and without regard for certain factors which interfered somewhat with the accuracy of the results. Experiments were begun the day following the operation without regard to any possible postoperative effect upon absorption. No attempt was made to make the amounts fed uniform. The appetites of the dogs are often capricious.

In order to make certain of as large a food intake as possible during an experiment, the animals were given at a feeding as much food as would be taken.

#### ILLUSTRATIVE PROTOCOLS.

*Dog 1.*—Young adult female.

Dec. 21, 1915. Weight 14 kilos.

Dec. 22. Operation. Partial pancreatectomy. Corpus and processus lienalis, 20 cm. in length and weighing 16.5 gm., extirpated. Processus uncinatus, 9.4 cm. long, transplanted under skin of abdomen and a fistula made.

Dec. 25. Graft is noticed for the first time to be discharging. Dog appears well. No sugar in urine. Fed milk and ground pancreas.

Dec. 29. Dog ate ravenously. Some reddening of skin near opening in graft.

Dec. 30. Fistula closed.

Dec. 31. Dog appears ill. Tissues about graft distended; on opening with knife about 15 cc. of thin sanguineous fluid obtained which digests casein.

Jan. 2, 1916. On incising swollen area about graft 60 cc. of thin sanguineous fluid escaped.

Jan. 3. Weight 12.3 kilos. Fistula discharging thin watery fluid. Urine does not reduce Fehling's solution.

Jan. 5. *Metabolism experiment begun.* 559.8 gm. of food eaten, containing 30 gm. of charcoal.

Jan. 6. Weight 11.9 kilos. 647.5 gm. of food eaten. Large fatty stool. A few grams do not contain charcoal, and these are rejected. Weight of stool admixed with charcoal, 529.5 gm. Stools contain numerous muscle fibers, of which about half retain their striations.

Jan. 7. Weight 11.9 kilos. Dog seems well. Ate 948.6 gm. of food. Stools weigh 436 gm.

Jan. 8. Ate 948 gm. of food.

Jan. 9. Weight 11.6 kilos. No food fed. Fistula was discharging throughout experiment. Stools weigh 676 gm.

Jan. 10. Stools weigh 31 gm. Fed milk, bread, and 60 gm. of charcoal.

Jan. 11. Stool black with charcoal.

Jan. 19. Operation. Subcutaneous transplant removed. It measured 6.6 by 3.1 by 1.9 cm. At the first operation the graft was 9 cm. long, but fully 2 cm. of this was left projecting beyond the skin incision and was lost by desiccation and necrosis. Aside from some increase in firmness the pancreatic tissue of the graft appears normal.

Jan. 20. Dog died of peritonitis.

*Metabolism Experiment. Jan. 5 to 8 (4 Days).*

Food: Moist weight	3,104 gm.	Dry weight	1,336 gm.
Nitrogen:	71.5 "	5.35 per cent.	
Fat:	431.1 "	32.3 "	" "
Feces: Moist weight	2,052.5 "	Dry weight	699.5 gm.
Nitrogen:	39.0 "	5.6 per cent.	
Fat:	313.1 "	44.8 "	" "
Nitrogen absorbed:	45.37 per cent;	lost 54.63 per cent.	
Fat	"	27.37 "	" " 72.63 " "

*Dog 2.*—Adult male. Weight 15.1 kilos.

Feb. 11, 1916. Operation. Pancreas extirpated except processus uncinatus. This measured 5.6 cm. in length and was planted into the anterior abdominal wall and a permanent fistula made. The pancreatic tissue removed weighed 22 gm.

Feb. 12. Dog lively. Graft healthy.

Feb. 13. Fed 300 cc. of milk.

Feb. 14. Weight 13.85 kilos.

Feb. 15. Dog appears sick. Fistula not discharging.

Feb. 16. Dog active. Drank 500 cc. of milk. Fistula secretes a thin fluid admixed with blood. No sugar in urine.

Feb. 17. Weight 13.9 kilos. *First metabolism experiment begun.*

Feb. 18. Graft secreting actively.

Feb. 19. Weight 13.7 kilos. Feces partly formed, partly semisolid. They contain flakes resembling butter in appearance and consistency. Microscopic examination reveals many well preserved muscle fibers and fat globules.

Feb. 20. Dog lively. Fistula secreting freely.

Feb. 21. Weight 13.2 kilos. Stools slate-gray, fatty.

*Metabolism Experiment 1. Feb. 17 to 21 (5 Days). Subcutaneous Graft. Extirpation of Remainder of Pancreas.*

Food: Moist weight	1,765 gm.	Dry weight	610 gm.
Nitrogen:	37.9 "	6.22 per cent.	
Fat:	175.7 "	28.8 "	" "
Feces: Moist weight	521.5 "	Dry weight	196 gm.
Nitrogen:	16.9 "	8.6 per cent.	
Fat:	43.6 "	22.2 "	" "
Nitrogen absorbed:	21.12 "	43.96 "	" "
Fat	"	132.1 "	75.20 " "

Feb. 22. No food given.

Feb. 24. Weight 12.96 kilos. Operation for removal of subcutaneous graft, which was firmly adherent to the surrounding tissues. Considerable blood lost



in cutting the adhesions, showing a large new blood supply to the graft. The excised pancreatic tissue was of normal appearance; the subcutaneous part was of firmer consistency than the intra-abdominal portion.

Feb. 25. Weight 11.8 kilos. Wound clean. *Second metabolism experiment started.* Ate 438.5 gm.

Feb. 26. Weight 11.2 kilos. Stool yellow, semisolid, and fatty, with rancid odor. Ate 9 gm. of food.

Feb. 27. Weight 11.2 kilos. Ate 91 gm. of food. Stool on microscopic examination found to contain many muscle fibers and much fat in the form of globules.

Feb. 28. Dog found dead in cage.

*Autopsy.*—No cause for death found. No pancreatic remnants in the abdomen or at the site of the pancreatic transplant in the abdominal wall. Stomach and small intestine empty. 58.5 gm. of fecal material were present in the colon and were added to the stools for analysis.

*Metabolism Experiment 2. Feb. 25 to 27 (3 Days).*

Food: Moist weight 539 gm. Dry weight 160.0 gm.

Nitrogen: 10.88 " 6.8 per cent.

Fat: 43.60 " 27.3 " "

Feces: Moist weight 232.5 " Dry weight 96.5 gm.

Nitrogen: 7.68 " 7.9 per cent.

Fat: 23.85 " 24.7 " "

Nitrogen absorbed: 3.20 " 29.41 " "

Fat " 19.75 " 45.3 " "

*Dog 3.*—Mar. 9, 1916. Weight 16.5 kilos. Operation. Subcutaneous transplant of processus uncinatus which was 10 cm. long. Remainder of pancreas, weighing 27 gm. extirpated.

Mar. 13. Good recovery from operation. Graft began to secrete.

Mar. 14. Weight 15.2 kilos. Dog lively. Fistula discharges a thin colorless fluid. *First metabolism experiment begun.*

Mar. 15. Weight 14.7 kilos.

Mar. 16. Weight 14.9 kilos. Stool semisolid, fatty, and contains a great number of muscle fibers and fat drops. Weight of feces 356 gm.

Mar. 17. Weight 14.9 kilos. Dog lively. Stools light yellow and of rancid odor; weight 411 gm.

Mar. 18. Weight 15.0 kilos. Fistula secreting. No food today. Stools weigh 173 gm.

Mar. 19. Stools 10 gm. Fed milk, bread, and charcoal.

Mar. 20. Weight 14.6 kilos. Stools black with charcoal.



*Metabolism Experiment 1. Mar. 14 to 17 (4 Days).*

Food: Moist weight	2,110.5	gm.	Dry weight	765.0	gm.
Nitrogen:	45.08	"	5.9	per cent.	
Fat:	215.88	"	28.2	"	"
Feces: Moist weight	1,161.5	"	Dry weight	417.5	gm.
Nitrogen:	28.81	"	6.9	per cent.	
Fat:	164.29	"	39.4	"	"
Nitrogen absorbed:	16.27	"	36.1	"	"
Fat	"	"	23.9	"	"

Mar. 21. Weight 14 kilos. Operation for removal of subcutaneous graft. The pancreatic remnant appeared normal.

Mar. 22. Weight 13.5 kilos. *Second metabolism experiment begun.* Ate 807.5 gm. of food. Dog appears in good condition but lies in cage.

Mar. 23. Dog lies in cage. Ate 77 gm. of food. Urine (480 cc.) reduced Fehling's solution. Stool 245 gm., semisolid, dark with charcoal. 3 gm. of stool not containing charcoal discarded.

Mar. 24. Dog much more lively. Stools weigh 143 gm.; the greater part admixed with charcoal. They are semisolid and microscopically show large numbers of well preserved muscle fibers and fat droplets.

Mar. 25. Dog very active. Stools weigh 284 gm.; diarrheal, light yellow.

Mar. 26. No food today. Stools weigh 113 gm.

Mar. 27. Weight 13.5 kilos. Feces 10 gm. Milk, bread, and charcoal fed. Dog appears well.

*Metabolism Experiment 2. Mar. 22 to 25 (4 Days).*

Food: Moist weight	1,786.0	gm.	Dry weight	575.0	gm.
Nitrogen:	34.5	"	6.0	per cent.	
Fat:	176.18	"	30.6	"	"
Feces: Moist weight	1,172	"	Dry weight	397.5	gm.
Nitrogen:	25.21	"	6.3	per cent.	
Fat:	145.19	"	36.5	"	"
Nitrogen absorbed:	9.29	"	26.9	"	"
Fat	"	"	17.6	"	"

Mar. 28 to Apr. 8. The dog became progressively weaker and after Apr. 2 was unable to stand. A severe and persistent glycosuria developed within 24 hours after the removal of the pancreatic remnants. Apr. 8. Killed with chloroform. No pancreatic tissue found in the abdominal cavity.

*Dog 4.—Adult male dog.*

Apr. 13, 1916. Weight 14.4 kilos. Entire pancreas removed except the processus uncinatus and lower part of the corpus pancreatis, which were transplanted with the main duct into the subcutaneous pocket.

Apr. 16. Recovery uneventful. No sugar in urine. Fed milk.

Apr. 17. *First metabolism experiment begun.* Weight 13.2 kilos. Fistula

secretes a clear colorless fluid. Ate 819 gm. of food. It contained 35 gm. of charcoal. Dog lively.

Apr. 18. Dog vomited; experiment stopped.

Apr. 19. Fed milk. Dog appears well.

Apr. 20. *First metabolism experiment recommenced.* Weight 13.4 kilos. Fistula discharges copiously. Ate 745.5 gm. of food.

Apr. 21. Food 375 gm.

Apr. 22. Dog well. Food 748 gm. Stool 163 gm. The skin about the fistula is reddened and excoriated.

Apr. 23. Dog lively. Fistula secreting. Food 799 gm. Stool 202 gm.; light yellow, and semisolid; contains abundance of muscle fibers and fat droplets.

Apr. 24. Weight 13.35 kilos. No food today. Stool 155 gm.

Apr. 25. Stool 25 gm. Food: milk, bread, and charcoal.

Apr. 26. Part of stools contains no charcoal. This portion weighs 116 gm. and was saved for analysis.

*Metabolism Experiment 1. Apr. 20 to 23 (4 Days).*

Food: Moist weight	2,668	gm.	Dry weight, 1,052.5 gm.
Nitrogen:	46.31	"	4.4 per cent.
Fat:	312.80	"	29.7 " "
Feces: Moist weight	781.0	"	Dry weight 260.0 gm.
Nitrogen:	16.12	"	6.2 per cent.
Fat:	80.42	"	30.9 " "
Nitrogen absorbed:	30.19	"	65.2 " "
Fat	"	232.38	" 74.3 " "

Apr. 27. Food: milk.

Apr. 28. Weight 12.3 kilos. *Second metabolism experiment started.* Fistula secreting actively. Throughout the experiment the dog wore a muzzle covered with tin in order to prevent licking of the fistula. The pancreatic transplant secreted freely.

Apr. 30. Stools are semisolid. Microscopically there are many well preserved muscle fibers and fat droplets.

May 2. Weight 12.9 kilos. No food.

May 3. Stool 197 gm. Dog well. Fed milk, bread, and charcoal.

*Metabolism Experiment 2. Apr. 28 to May 1 (4 Days).*

Food: Moist weight	3,582	gm.	Dry weight 1,495 gm.
Nitrogen:	78.49	"	5.3 per cent.
Fat:	488.57	"	32.7 " "
Feces: Moist weight	2,018	"	Dry weight 713.5 gm.
Nitrogen:	43.74	"	6.1 per cent.
Fat:	257.65	"	36.1 " "
Nitrogen absorbed:	34.75	"	44.3 " "
Fat	"	230.92	" 47.3 " "

May 5. Weight 12.00 kilos.

May 7. Fistula secretes 0.5 cc. of fluid per minute. One drop of the fluid digests 5 cc. of 1 per cent casein in 4 hours.

May 8. Weight 11.35 kilos. Dog is growing weaker. He eats ravenously.

May 8 to 19. Dog remains well. He is fed raw pancreas of three pigs, with chopped horse flesh and 500 cc. of milk daily. A gain in weight from 11.35 to 12.5 kilos occurred. The secretion of the fistula has gradually diminished.

May 19. Weight 12.5 kilos. The dog is in good condition but looks thin. Removal of the subcutaneous graft.

May 20. Weight 11.6 kilos. Dog lively and appears well. Urine, 370 cc., contains 18.5 gm. of glucose. *Third metabolism experiment started.*

May 21. Weight 11.15 kilos. Dog apathetic, but does not appear sick. Ate 441 gm. of food. No stools.

May 22. Food 380 gm. Stools have rancid odor and all contain charcoal; weight 131 gm.

May 23. Weight 11.15 kilos. Dog lively. Food 285 gm. Feces weigh 222 gm., 110 gm. of which contain much charcoal. On microscopic examination found to contain many muscle fibers and fat droplets.

May 24. Food 673 gm. Feces 266 gm.; partly formed, partly semisolid.

May 25. Weight 10.65 kilos. No food allowed. Stools 358 gm.

May 26. Fed milk, bread, and 30 gm. of charcoal. Stools 94 gm.

*Metabolism Experiment 3. May 20 to 24 (5 Days).*

Food: Moist weight	1,836	gm.	Dry weight	717.5	gm.
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Nitrogen:	37.88	"	5.3	per cent.
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Fat:	213.67	"	29.8	"	"
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Feces: Moist weight	1,071	"	Dry weight	413	gm.
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Nitrogen:	28.13	"	6.8	per cent.
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Fat:	155.45	"	37.6	"	"
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Nitrogen absorbed:	9.76	"	25.8	"	"
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Fat	"	58.22	"	27.2	"	"
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The dog lost 600 gm. during this experiment. He emaciated rapidly and became progressively weaker.

May 27. Weight 10.6 kilos. All the stools contain much charcoal. Urine, 650 cc., contains 81.25 gm. of glucose. *Fourth metabolism experiment started.* Lard fed instead of butter. Food 1,099 gm.

May 28. Weight 10 kilos. Dog stands steadily and seems well. Food 926 gm. Feces 409 gm.

May 29. Weight 9.9 kilos. Dog appears well. Food 747 gm. Feces 227 gm.; light yellow, semisolid.

May 30. Weight 9.9 kilos. Food 677 gm. Feces 532 gm.

May 31. Weight 9.75 kilos. Dog found lying in cage, but arises when approached; unsteady on his feet. Feces 440 gm.

June 1. Weight 9.2 kilos. No food today. Dog very weak. Stool 70 gm.

*Metabolism Experiment 4. May 27 to 31 (5 Days).*

Food: Moist weight	3,449 gm.	Dry weight	1,402.5 gm.
Nitrogen:	73.35 "	5.2 per cent.	
Fat:	483.72 "	34.5 " "	
Feces: Moist weight	1,678 "	Dry weight	664 gm.
Nitrogen:	37.18 "	5.6 per cent.	
Fat:	304.31 "	45.8 " "	
Nitrogen absorbed:	36.17 "	49.3 " "	
Fat	" 179.41 "	37.1 " "	

June 2. Weight 9.1 kilos. Stands very unsteadily. Lies in cage unless some one approaches him. Etherized. No pancreatic remains found in the abdomen.

*Dog 5.*—Female pup.

Mar. 16, 1916. Weight 4.8 kilos. Entire pancreas except processus uncinatus removed. The latter was left *in situ*. It measured 5 by 2.5 cm. The pancreatic tissue is separated from the duodenum by mesentery.

Mar. 17. Dog lively. Fehling's solution not reduced by urine.

Mar. 23. Dog drinks milk and eats meat; seems well.

Mar. 26. Weight 3.5 kilos.

Mar. 27 to Apr. 8. Fed raw pancreas ground up with cooked horse flesh and milk.

Apr. 9. Fed only milk.

Apr. 10. Weight 4.8 kilos. *First metabolism experiment begun.* Dog in good condition. Food eaten 209.5 gm., containing 20 gm. of charcoal.

Apr. 11. Food 558 gm. Large part of feces contains charcoal; weight 108 gm. Portion free from charcoal easily separated.

Apr. 12. Food 651.6 gm. Feces 122 gm.; dark gray, formed, and found to contain on microscopic examination large amounts of muscle fibers and fat droplets.

Apr. 13. Food 239 gm. Feces 158 gm.; semisolid, yellow, fatty.

Apr. 14. Food 260 gm. Feces 123 gm.

Apr. 15. Fed 300 cc. of milk with charcoal. Feces 57 gm. Dog lively.

Apr. 16. Feces, 22 gm., unmixed with charcoal.

Apr. 17. Weight 4 kilos. Fed raw pancreas with chopped cooked horse flesh and milk. All the feces contain charcoal.

*Metabolism Experiment 1. Apr. 10 to 14 (5 Days).*

Food: Moist weight	1,709 gm.	Dry weight	550 gm.
Nitrogen:	32.01 "	5.8 per cent.	
Fat:	150.9 "	27.4 " "	
Feces: Moist weight	592 "	Dry weight	220 gm.
Nitrogen:	14.98 "	6.8 per cent.	
Fat:	72.07 "	32.8 " "	
Nitrogen absorbed:	17.98 "	56.2 " "	
Fat	" 77.8 "	51.6 " "	

June 1. Weight 4.8 kilos. Urine contains sugar for the first time. Since Apr. 15 the dog has been given raw pig's pancreas daily.

June 29. Weight 4.1 kilos. Urine reduces Fehling's solution.

July 26. Found dead this morning. Weight 2.9 kilos.

*Autopsy.*—Dog greatly emaciated. All internal organs seem free from disease. A piece of tissue resembling a lymph node lies near the lower end of the duodenum in the mesentery. It is about 1 cm. in diameter and the thicker portion is surrounded by a zone of thinner tissue making the whole mass measure 3 by 1 cm. On histological examination this was found to be the atrophied and sclerosed pancreatic remnant.

### *Fat Absorption in Partially Depancreatized Dogs.*

Metabolism experiments were done on four partially depancreatized dogs with subcutaneous transplants, which discharged the pancreatic juice externally. The animals were free to lick their fistulas and thus ingest pancreatic juice. How much pancreatic secretion was obtained in this way could not be determined. The amounts and percentages of fat absorbed are given in Table I.

TABLE I.

*Fat Absorption in Partially Depancreatized Dogs in Which All Pancreatic Juice Was Excluded from the Intestine.*

Dog.	Weight.*	Fat ingested.		Fat absorbed.			Remarks.
		Total amount.	Amount per kilo.	Total amount.		Amount per kilo.	
	kg.	gm.	gm.	gm.	per cent	gm.	
1	11.9	431.12	36.1	118.0	27.37	9.88	Subcutaneous transplant; remainder of pancreas extirpated.
2	13.5	175.68	13.0	132.1	75.2	9.78	
3	14.9	215.88	14.5	51.6	23.9	3.47	
4	13.4	312.8	23.38	232.38	74.3	17.4	Processus uncinatus left <i>in situ</i> ; remainder of pancreas extirpated.
5	4.4	150.9	34.28	77.8	51.6	17.66	

\*The weights given are the averages of the daily weights of each dog.

Dogs with functioning subcutaneous grafts with the external secretion discharging freely may absorb no more fat than a dog in which the ducts are occluded and the gland undergoing rapid and progressive atrophy. This is seen by comparing the results of the experiments on the first four dogs (Nos. 1, 2, 3, and 4) with those on Dog 5, presented in Table I. In Dog 5 the processus uncinatus



was left in the abdomen and the remainder of the pancreas removed. There was no communication between the pancreatic remnant and the lumen of the duodenum; the dog received neither pancreatic juice nor pancreas in its food, and yet this animal absorbed 51.6 per cent of the fat ingested. The mean average of the percentages absorbed by the other four animals is 50.19 per cent. Dog 5 absorbed 17.66 gm. of fat per kilo of body weight. 17.38 gm. was the maximum per kilo absorbed by any of the other animals, in the tabulated results.

The fact that the fat absorption in the dogs with atrophied pancreas, studied by Pratt, Lamson, and Marks, was less on the average than in our dogs with pancreatic transplants which were discharging pancreatic juice freely, would seem at first sight to support Lombroso's view that absorption varied with the condition of the pancreatic remnant. But in Dog 5 with atrophying pancreas, as we have shown, the absorption was equal to that in the dogs with subcutaneous transplants that were not undergoing rapid atrophy. The same food mixture was given to all the animals in the present investigation. In the earlier study of Pratt, Lamson, and Marks a different food mixture was fed and it may have been less easily absorbed. However, there is no doubt that different animals when deprived of their pancreatic juice vary greatly in their power to absorb fat, and furthermore, that there is a great variation in the same animal on the same diet at different times. This was shown in the experiments of Pratt, Lamson, and Marks.

In the present study Dogs 2 and 4 absorbed the relatively large percentages of 75.2 and 74.3 per cent of the fat ingested, while Dogs 1 and 3 absorbed but 27.37 and 23.9 per cent.

Much more secretion flowed from the cutaneous fistula in Dog 4 than from the fistulas of the other animals. A proteolytic ferment was demonstrated in the secretion. The dog was seen to lick the fistula frequently and it is probable that the animal ingested more secretion than did the other dogs. Nevertheless, the percentage of fat absorbed by Dog 4 was no greater than that absorbed by Dog 2.

The number of grams of fat absorbed per kilo of body weight varied in the different dogs. No relation existed between the number of grams of fat ingested and the amount absorbed.



Burkhardt, Fleckseder, and Lombroso carried out metabolism experiments on dogs with pancreatic cutaneous fistulas. Experiments were made during two periods, one in which the fistulas were licked and one in which the licking was prevented. Conflicting results were obtained.

Fleckseder and Lombroso concluded from their experiments that, since it made little difference in the absorption of fat whether or not the animal obtained the pancreatic secretion from the pancreatic transplant, the absorption must be governed by an internal function of the pancreas. We have repeated the experiments on one dog. The results are given in Table II.

TABLE II.

*Fat Absorption in Dog 4 with and without Licking of Pancreatic Juice.*

Experiment No.	Date.	Weight.* kg.	Fat ingested.		Fat absorbed.			Remarks.
			Total amount.	Amount per kilo.	Total amount.		Amount per kilo.	
			gm.	gm.	gm.	per cent	gm.	
1	Apr. 20 to 23	13.37	312.80	23.38	232.38	74.30	17.38	Fistula licked.
2	" 28 " May 1	12.60	488.57	38.77	230.92	47.3	18.32	Licking prevented; dog muzzled.

\* The weights given are the averages of the daily weights during each experiment.

Dog 4 in the first metabolism experiment was permitted to lick the fistula and thus obtain the pancreatic juice. In the second experiment the dog was prevented from licking the fistula by means of a muzzle covered with tin. The fistula in this animal continuously secreted enough fluid to bathe the entire ventral parts of the abdomen and chest. The skin over these areas showed evidences of being digested. The secretion from the fistula contained trypsin.

In the first experiment 74.3 per cent of the ingested material was absorbed. After muzzling the dog the absorption dropped to 47.3 per cent. This increased loss in the feces of 27 per cent of the fat ingested suggests that the pancreatic secretion increased the amount of fat absorbed. This view is supported by the fact that the feces contained 5.2 per cent more fat. The total amount of fat absorbed, however, was nearly the same in the two experiments, and the amount of fat absorbed per kilo was actually greater when the dog was prevented from licking the fistula. As the dog was not muzzled

until the experiment was begun, it is possible that pancreatic secretion ingested previously influenced somewhat the absorption of fat.

It would not be surprising if the pancreatic secretion introduced *per os* had increased somewhat the absorption of fat, because Pratt, Lamson, and Marks observed a greater absorption after the feeding of pancreatic ferments to dogs in which the pancreas had been separated from the duodenum.

Lombroso believed that a definite relation existed between the state of preservation of the pancreatic remnant in his dogs and the amount of fat absorbed. This conclusion was based upon the finding of degenerated pancreatic remnants in dogs showing a poor fat absorption. But his own findings and those of other investigators do not support this view. In the first metabolism experiments following partial pancreatectomy, one of his dogs absorbed 46 per cent (48 gm.), and another dog absorbed none of the fat ingested. 2 weeks later the same animals absorbed 68 per cent (66 gm.) and 16 per cent (13 gm.). There is no reason to assume that the pancreatic remnant was in a better state of preservation 2 weeks after the first metabolism experiments.

Two dogs, reported by Rosenberg, absorbed from 49.06 to 84 per cent of the fat ingested, at a time when the pancreas had undergone almost complete fibroid degeneration. Sandmeyer studied the fat absorption in a partially depancreatized dog in which he believed no communication existed between the pancreas and duodenum. The percentages of fat lost in the stools of this dog varied from 9.1 to 163.5 per cent. The greatest fat losses were not confined to any one period, but occurred from time to time throughout the investigation. Hence, they could not be attributed to degeneration of the pancreatic remnant.

Dogs 1 and 3 absorbed but 27.37 and 23.9 per cent. These low percentages of fat absorption could not be ascribed to degenerative changes in the pancreatic remnants as maintained by Lombroso, because when these were removed at the second operation they were found to be well preserved.

#### *Fat Absorption in Completely Depancreatized Dogs.*

At a second operation the pancreatic remnant was removed from Dogs 2, 3, and 4. Fat absorption was then studied in these animals. The results are given in Table III.

TABLE III.

*Fat Absorption in Completely Depancreatized Dogs.*

Experiment No.	Dog.	Weight.*	Fat ingested.		Fat absorbed.		
			Total amount.	Amount per kilo.	Total amount.		Amount per kilo.
		kg.	gm.	gm.	gm.	per cent	gm.
2	2	11.4	43.6	3.82	19.75	45.3	1.73
2	3	13.4	176.18	13.11	30.99	17.59	3.05
3	4	11.2	213.67	19.00	58.22	27.24	5.17
4	4	10.1	483.72	48.22	179.41	37.1	17.88

\* The weights given are the averages of the daily weights of each dog.

Dog 2 was in poor condition during the metabolism experiment and died before its completion. The animal ingested but 43.6 gm. of fat. Nevertheless, 45.3 per cent of this was absorbed.

Each dog absorbed a considerable amount of fat, although the percentages, the actual amount, and the number of grams per kilo of body weight varied much in the different animals and in the two experiments on the same animal. As the autopsies on these dogs showed that all pancreatic tissue had been extirpated, the experiments prove that fat may be absorbed from the intestine by depancreatized dogs in relatively large amounts, even when the fat fed is not in the form of an emulsion. Dog 4 absorbed 37 per cent of fat fed, chiefly in the form of lard, 9 to 12 days after the removal of all pancreatic tissue. In the other experiments butter was given. Since the publication of Abelman's studies in 1890, it has been generally held that a depancreatized dog can absorb only the fat of milk.

A comparison of the fat absorbed during the stage of partial depancreatization and after complete pancreatectomy is given in Table IV.

After partial depancreatization Dog 2 absorbed 75.2 per cent of the fat ingested (Metabolism Experiment 1), while 45.3 per cent was absorbed after complete pancreatectomy. Results almost identical to these were obtained in the first and second metabolism experiments on Dog 4, although the transplant was still secreting in the second experiment. The only difference is that the dog obtained no secretion, as it was muzzled. It will be seen that Dog 2 absorbed a larger percentage of fat when completely depancreatized

TABLE IV.

*Comparison of Fat Absorption before and after Complete Depancreatization.*

Experiment No.	Dog.	Date.	Weight.*	Pancreatectomy.	Fat ingested.		Fat absorbed.			Remarks.
					Total amount.	Amount per kilo.	Total amount.		Amount per kilo.	
			kg.		gm.	gm.	gm.	per cent	gm.	
1	1	Jan. 5 to 8	11.9	Partial.	431.12	36.10	118.0	27.4	9.88	Fistula licked.
1	2	Feb. 17 to 21	13.5	Partial.	175.68	13.0	132.1	75.2	9.78	Fistula licked.
2		" 25 " 27	11.4	Complete.	43.60	3.82	19.75	45.3	1.73	
1	3	Mar. 14 to 17	14.9	Partial.	215.88	14.5	51.6	23.9	3.47	Fistula licked.
2		" 22 " 25	13.4	Complete.	176.18	13.11	30.99	17.6	3.05	
1	4	Apr. 20 to 23	13.4	Partial.	312.8	23.38	232.38	74.3	17.4	Fistula licked.
2		" 28 " May 1	12.6	"	488.56	38.77	230.92	47.3	18.32	Muzzled.
3		May 20 " 24	11.2	Complete.	213.67	19.00	58.22	27.2	5.17	
4		" 27 " 31	10.0	"	483.72	48.22	179.41	37.1	17.88	

\* The weights given are the averages of the daily weights throughout an experiment.

than did Dog 4 which possessed functioning pancreatic tissue. These findings are decidedly against Lombroso's theory that absorption is controlled by an internal secretion of the pancreas. Dogs 3 (Metabolism Experiments 1 and 2) and 4 (Metabolism Experiments 2 and 4) absorbed but 6.3 and 10.2 per cent less fat after complete depancreatization than after partial extirpation of the gland. Greater differences in the percentage absorption of fat in the same animal are reported by Lombroso, and Pratt, Lamson, and Marks, in their studies on partially depancreatized dogs.

The number of grams of fat absorbed per kilo of body weight varies in experiments on the same dog and on different dogs, as is shown in Table IV. Dog 2 absorbed much less fat after complete than after partial depancreatization. But it will be noted that much less fat was ingested. A similar result occurred in the first metabolism experiment following complete depancreatization in Dog 4.

The poorer absorption in these dogs may have been a result of the operation. The experiments were begun the day following. However, under the same experimental conditions Dog 3 did not show an appreciable increase in the fat lost in the feces. In the first experiment Dog 3 absorbed 3.47 gm. of fat per kilo of body weight. At this time the dog was partially depancreatized and was allowed to lick the secretion from the fistula. After complete depancreatization 3.05 gm. of fat per kilo were absorbed. After complete extirpation of the gland, Dog 4 (Metabolism Experiment 4) absorbed but 0.44 gm. less fat per kilo than after partial depancreatization (Metabolism Experiment 2). In the latter experiment the dog possessed a cutaneous pancreatic fistula, but was muzzled so that no pancreatic secretion could be ingested. In Metabolism Experiment 4, 0.5 gm. more fat was absorbed per kilo than in the first metabolism experiment, in which the dog licked the pancreatic fistula. These findings show that it is possible for a completely depancreatized dog to absorb as much fat per kilo of body weight as when the same dog possesses functioning pancreatic tissue which does not secrete into the intestines.

#### CONCLUSIONS.

It was found that dogs with a subcutaneous transplant secreting and discharging pancreatic juice externally absorbed no more fat than dogs in which the pancreatic remnant was undergoing rapid atrophy and sclerosis. Hence, the condition of the pancreatic tissue remaining in the body does not influence the amount of fat absorbed by the intestine.

The absorption of fat by the intestinal mucous membrane is always markedly disturbed when the pancreatic secretion is excluded from the intestine.

After the complete removal of all pancreatic tissue from an animal, the absorption of considerable amounts of fat can still take place.



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## SOME FIELD EXPERIMENTS BEARING ON THE TRANSMISSION OF BLACKHEAD IN TURKEYS.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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During the spring and summer of 1916 when the Department buildings and grounds were not yet available, a small piece of land on the campus of Princeton University was put at our disposal by the University authorities. On this land the experiments to be described were carried out. Although they are incomplete on account of restricted facilities, it seems desirable to publish them now, rather than wait another year, in order to stimulate others to utilize the more positive features of the work and thereby save a year in testing the final practicability of the suggestions made.

To obtain animals free from infection, for experimental purposes, it was necessary to rear them, as was done in 1913 and 1914, in the incubator and brooder.<sup>1</sup> To test again at the same time the presence or absence of the parasite of blackhead in eggs, these were obtained from three different farms on all of which the disease had prevailed during the preceding season, if we are to accept mortality as an indicator. The positive demonstration of blackhead on two of the farms was made by autopsies in the course of the season.

Since the artificial incubation and brooding of turkeys is not practised at the present time, a few details of the procedure as actually followed may not be amiss, although some of these details have been referred to in an earlier paper.<sup>1</sup> Just how far the precautions exercised will be necessary in practice must be worked out later. In the work before us, the main task was to obtain a lot of healthy animals. Any failure meant the postponement of further work for a whole year.

<sup>1</sup> Smith, T., *J. Med. Research*, 1915, xxxiii, 243.

After collection, the eggs were kept in a cool place and turned once a day. For transportation to Princeton, they were wrapped in cotton-wool, boxed, and carried in the hand. No time was lost in starting incubation. As a preliminary precaution, they were washed and gently scrubbed with warm water, dipped in warm 0.5 per cent mercuric chloride for 30 seconds, then washed in warm boiled water, and dried with sterilized towels. When placed in the incubator, April 21, 1 egg was 11 days old, 10 were 6 days old, 11 were 5 days old, 7 were 4 days old, 9 were 3 days old, 3 were 2 days old, and 1 was 1 day old. 1 was found cracked later on and rejected, leaving 41 eggs in all.

The incubator used was an ordinary one with a capacity of 144 eggs. The kerosene lamp was replaced by a Bunsen burner. Three shallow pans were supported on rods in the bottom of the egg chamber and in these water was kept throughout the period of incubation. Moisture was not supplied in any other way. The eggs were turned twice a day but were not otherwise handled. All the turkeys hatched on May 18 in the course of 24 hours.

From Source A, 24 eggs were incubated, 22 hatched. The remaining 2 eggs contained full grown embryos.

From Source B, 5 eggs were incubated, 2 hatched, 1 failed, and 2 were sterile.

From Source C, 12 eggs were incubated, 6 were sterile, 3 hatched. 2 had full grown embryos and 1 a partly macerated small embryo.

The results would perhaps have been better under other than improvised surroundings. The incubator temperature rose to 107° F. during the night of the 1st and 2nd day, and it was feared that the embryos had been destroyed. On the whole, the output was as large as could be handled under the circumstances.

During the 1st week four poults with weak legs were killed because they were unable to feed themselves properly. There were thus left twenty-three vigorous poults from forty-one eggs. On May 19, they were moved about a mile and placed in a brooder on a house porch, protected against rain, where they could be closely watched and properly fed. On June 1, brood and brooder were again carried some distance to an open, wire-screened shelter about 9 feet by 12 feet with a sloping canvas top to keep the rain off. This shelter

was placed on a lawn directly behind the laboratory building (Guyot Hall of Princeton University) where the Department was housed during the year. The southern exposure and proximity to the buildings made the shelter at times extremely hot during the summer. Rain was not entirely excluded since the screened sides were not protected. The position of the shelter was, however, such that it was protected from driving rains. On June 17, 30 days after the hatching, the brooder was removed from the shelter and from that time on the turkeys spent the day in a yard staked out with poultry wire which was occasionally shifted. The yard covered, roughly speaking, about one-eighth of an acre. Sparrows and song birds visited the grounds daily. During the night and on rainy days the turkeys were locked in the shelter.

Up to July 3, 46 days after hatching, the twenty-three turkeys had not had any illness. They were all vigorous and without visible deformities.

So far, the result of the work was clear. Twenty-three turkeys were carried along for more than 6 weeks without death or disease. This confirms on a larger scale the work done previously. It shows that healthy turkeys may be raised from the eggs of infected flocks when all older turkeys and poultry are kept away and the ground has not been used before. There is thus no evidence favoring the theory that the protozoan parasite can be transmitted in the egg. The experiment also shows that young turkeys are more resistant than is usually assumed. This flock was handled and moved about considerably and was not kept in a closed shelter at night after the 1st month. The want of resistance exhibited by young turkeys must, at least in part, be ascribed to latent infection waiting on some depression of vitality for multiplication in the tissues. In this resistance the food probably plays a considerable but as yet unmeasured part. As there was no latent infection or an infected environment to be dealt with, the food actually given and to be described may not be satisfactory in the presence of infection. It should, therefore, be understood that no claim is made for the food or treatment in warding off disease, as there was none to guard against. It was, however, necessary to provide the best food available to counteract the influences of confinement.

The poults were fed about four times a day during the 1st month. During the first 3 days in the brooder, they received bread, scalded milk, boiled egg, and some lettuce. Thereafter they were fed with a mixture prepared and sterilized in glass jars and made up as follows: 500 gm. of chick feed and 1,000 cc. of milk plus 5 eggs, mixed thoroughly after heating in a double boiler, and then autoclaved. In addition they received sour milk and lettuce. On warm days they were allowed to move about on the lawn and obtain some grass. The food mixture was changed from time to time both in amount and composition, by replacing an increasing amount of cooked food with raw chick feed, until a relatively small per cent of the food was cooked. Later, coarser grain was substituted. The sour milk was continued during the summer and fall.

#### *Exposure to Domestic Fowls.*

The statement has been made from time to time that the parasite of this disease may be carried and disseminated by poultry. No conclusive experiments for or against this supposition have been made. Nor is it likely that any one, or even several tests can decide the question. It is conceivable that fowls in one geographical section or fowls of a given breed may act as agents. The writer's experience in 1910,<sup>1</sup> when incubator turkeys kept near a poultry yard died of blackhead, made a test of this possible source of infection seem most urgent.

On July 3, four incubator turkeys were segregated in a second enclosure for an experiment to be described later. On July 14, when the remaining nineteen turkeys were 57 days old, they were divided into two lots of nine and ten respectively. The screened shelter was divided into two sections by a partition. Two doors led to two different yards, separated by a lane at least 5 feet wide.

Two yearling hens (White Leghorn), from a farm having no turkeys, were penned with one lot of nine turkeys from July 14 until August 14—a period of 31 days—when the experiment was closed. None of the turkeys showed any indications of disease during this period or at the end of the experiment. It is probable that certain nematodes (*Heterakis*) found in the ceca of several turkeys later on came from the poultry.

Two yearling hens were penned with the second group of ten turkeys from July 17 to August 14, a period of 28 days. No disease or minor ailment appeared among the turkeys during this time. The hens used came from a farm on which

only 8 turkeys survived out of a lot of 57. The hens, of which there were about a hundred, had been with the turkeys until 2 weeks before the test.

The negative outcome of these experiments does not, of course, prove that poultry does not transmit the protozoa of blackhead. It is, however, distinctly encouraging from the practical standpoint. Experiments similar to this should be made in different sections and with different breeds of fowls upon incubator turkeys.

*Exposure to an Infected Adult Turkey on Non-Infected Ground.*

On August 14, the nineteen turkeys which had passed through the exposure to poultry and remained well were divided into three lots. Two of these were sent to Farms A and C on which blackhead had prevailed during the season, in order to test their susceptibility at this time. A third lot of nine was kept in the original enclosure and an infected turkey-hen (No. 120) penned with them. This hen had been raised on Farm B in 1915. In 1916, she hatched and brooded nine poults among which there were deaths from blackhead when the poults were 19, 33, 34, 35, and 37 days old, respectively. She had not been in contact with young turkeys since July 3. Before placing her with the incubator turkeys, the feet were thoroughly scrubbed to remove all attached soil.

Within 17 days there were signs of disease in the enclosure. One (No. 137) died of blackhead on September 9, 26 days after beginning of exposure. No. 139 was very ill on September 12 and chloroformed. Pronounced lesions of the liver and ceca were found. On September 15, No. 132 was very ill and chloroformed. Nos. 140 and 141 which were ill for a week, beginning August 31, recovered and are still alive and of normal size.

Exposure to an adult turkey-hen thus led to disease within 17 days. Three died, two recovered, and four did not show any disturbance of health.

*Exposure to Infected Adults on Infected Ground.*

Six incubator turkeys were sent to Farm C, on August 14, to be placed on infected ground and come in contact with adult turkeys. Of these, one died on August 29, one on August 31, and one on September 1. Three survived but no information is at hand whether they were manifestly ill at any time.

Four incubator turkeys were exposed to a similar environment on Farm A, beginning August 14. One died on August 28, one on August 30, and one on September 3. One survived.

In all cases the dead birds were sent to the laboratory and the diagnosis of blackhead was confirmed by autopsy and microscopic examination of fixed and hardened tissue.



*Exposure to Infected Young Turkeys.*

On July 3, when the incubator turkeys were 46 days old, four were taken from the main flock to be penned with young infected birds.

In order to do this it became necessary to stake out a second enclosure or yard. It was separated from the first enclosure by a lane about 15 feet wide. A brooder was placed in it to afford shelter at night and during storms. The openings were enlarged and covered with wire netting to keep rats and other vermin out.

From a small brood on Farm B in which blackhead had appeared, two young turkeys (Nos. 154 and 155) were placed with the four incubator turkeys into the enclosure on virgin soil. On July 7, three young turkeys (Nos. 167, 168, and 169) from another brood on Farm B, in which blackhead prevailed, were placed into the same enclosure. On July 11, No. 168, being very feeble, was killed. The wall of one cecum was locally thickened but there was no necrosis or exudation into the lumen. Sections showed subsequently that the thickening was due to the parasite of blackhead. On August 14, 38 days after the poults had been penned together, No. 169 appeared droopy, and was chloroformed. The walls of one cecum over the distal half were greatly thickened but there was no exudate. The liver was permeated with yellowish foci from 2 to 6 mm. in diameter. Sections of the liver and ceca showed the usual picture of this disease.

On this day (August 14) the experiment was closed because the shelter was becoming too small and conditions were unsatisfactory. There were remaining at this time the three originally exposed birds, Nos. 154, 155, and 167, all apparently well, and four incubator turkeys, all of which had remained well to date. Of this latter group one was killed the following day. No traces of disease could be found.

This enclosure had thus harbored two diseased turkeys, one for 4 (No. 168), the other for 38 days (No. 169). It is impossible to state when the latter became actively diseased.

Unless our turkeys were naturally immune—an assumption disproved by experiments detailed above—the experiment shows that a prompt transfer of infection from the diseased young stock did not occur, in spite of the fact that these birds were housed in the confined space of a small brooder every night and associated in the enclosure during the day. This result is in harmony with the experiments of 1914.<sup>1</sup> Eleven incubator turkeys were at that time kept with eight acutely diseased turkeys in a small shelter at night and in the same open enclosure by day. Only one became acutely diseased and died.

The ultimate fate of this lot of turkeys is of no significance. Owing to the necessity for closing this experiment on account of inadequate shelter and grounds, the surviving turkeys were moved to grounds on Farm B which had been cleared of turkeys early in July. Several

died of blackhead here. It again became necessary to move the remainder and they were placed with those exposed to the adult hen already referred to. Only one survived and is now in fine condition.

#### DISCUSSION.

Before drawing any conclusions based on this season's field experiments, it will be necessary to point out several weak points. To make the exposure tests more conclusive, a certain number of incubator turkeys should have been kept as controls throughout the summer. The turkeys exposed to the hens should have been kept the rest of the season and not used in any subsequent tests. It was, however, impossible to provide the necessary shelters and runs.

To meet the first criticism on the absence of controls, it may be stated that this disease does not have a long period of incubation. A large percentage of poults die between the end of the 2nd and 3rd weeks of life. Thus during the present season, the first death among the brood of one hen on Farm B occurred in 18 days, among the brood of the second hen in 23 days.

The fact that all the incubator turkeys remained well throughout the time of association with hens and became diseased as late as 2 weeks after the exposure to actual infection had begun does not make the assumption that they had acquired disease from the hens probable. If they did contract the disease from them it required over 6 weeks from the beginning of the exposure to bring it out.

Table I gives the details of the two experiments.

More of the turkeys that had been exposed to hens not in previous contact with diseased turkeys died than of the other lot. A superficial study might give the impression that the second lot had obtained an immunizing dose from the hens previously exposed to blackhead, for six out of nine of the first lot and but three out of ten of the second lot became openly diseased. The difference is, however, due to the fact that the mortality of the turkeys penned with Turkey-hen 120 was only one-third. This low percentage may be ascribed to the relatively diluted infection due to one bird on virgin soil as compared with that existing on farms where the soil may be infected and where there are several adults.

TABLE I.

Turkey No.	Exposure to hens from July 14 and 17 to Aug. 14.	Exposure to blackhead infection on Aug. 14.	Result.
126 }	Two hens from turkey-free farm.	Farm A.	Died on Aug. 28. Blackhead.
129 }		" "	Lived.
130 }		" C.	Died on Aug. 29. Blackhead.
131 }		" "	" " " 31. "
132 }		Turkey-hen 120.	Chloroformed on Sept. 15. Blackhead.
133 }		Farm A.	Died on Sept. 3. Blackhead.
134 }		" C.	Lived.
135 }		" "	Died on Sept. 1. Blackhead.
136 }		Turkey-hen 120.	Lived.
124 }		" 120.	"
125 }	Two hens from farm infected with blackhead.	" 120.	"
127 }		Farm C.	"
128 }		" A.	Died on Aug. 30. Blackhead.
137 }		Turkey-hen 120.	" " Sept. 9. "
138 }		" 120.	Lived.
139 }		" 120.	Chloroformed on Sept. 12. Blackhead.
140 }		" 120.	Lived.
141 }		" 120.	"
142 }		Farm C.	"

## SUMMARY.

It seems, therefore, pending further field tests on a larger territory, that the following facts are fairly well established by the above experiments.

1. Healthy turkeys may be raised in an incubator from eggs of infected birds. In the above experiments all remained well to August 14, the end of the hen exposure test, when they were 12 weeks and 4 days old. The first death occurred 2 weeks after the beginning of exposure to actual disease, when they were more than 14 weeks old.

2. Hens from a blackhead farm and from a farm free from turkeys did not convey the disease to the incubator turkeys on uninfected land.

3. The infection is either not transmitted at all or only under exceptional conditions by turkeys in the early acute stage. It is probably carried and shed by those birds which have successfully passed through an attack.

Any definite statement concerning the mode of transmission of the infection cannot be made. The vehicle is unknown. The nature of the disease makes it probable, however, that it is introduced with the food, that it lodges first in one or both ceca, and that fecal matter is the vehicle.

During the entire season, portions of the small intestines of all the turkeys that died or were killed were sectioned and examined both with reference to the possible presence of coccidia and of any preliminary stage of *Amæba meleagridis*. Sections were studied from the upper (duodenum), middle, and lower portions. A few coccidia cysts were found in two turkeys and are referred to more in detail elsewhere.<sup>2</sup> In a third turkey an intracellular parasite was seen which is very minute and which differs from those usually met with in birds. It is tentatively placed with the coccidia. It was not seen in any other case although searched for to obtain more material for study. It may be that we have an aberrant parasite to deal with which comes from the insects eaten and obtains lodgment in rare cases only. The existence of any earlier stages of the black-head parasites in the small intestine whence they move down into the ceca is contradicted by the focal lesions found in the ceca and by the fact that in many cases only one cecum is attacked and this only in a single, restricted area. If the parasites multiply higher up we should expect both ceca to become infected.

There is some evidence pointing to a greater resistance of older birds than is usually presented by young turkeys. Thus, the total mortality (including those chloroformed while sick) following exposure on the infected farms and to the yearling turkey-hens was nine out of nineteen exposed, or a trifle less than 50 per cent. This figure is usually exceeded among those exposed immediately after hatching. The surviving turkeys are still well at the present time (January 1917).

The other kind of evidence is derived from the histological examination of the lesions in the liver and ceca. In these organs the process was rather early associated with extensive infiltration of roundish cells, while the tendency to necrosis was relatively slight. The lesions

<sup>2</sup> Smith, T., and Smillie, E. W., *J. Exp. Med.*, 1917, xxv, 415.

of the walls of the ceca were characterized by a marked thickening of the wall with little or no necrosis and exudation of fibrin.

In repeating tests of this kind it will be well for the experimenter to bear in mind that they are no longer laboratory experiments but conform to natural occurrences. The infection is not isolated in pure culture but is associated in the body of the carrier with unknown factors, both normal and pathological. The virus, if such it may be called, may be mixed with different injurious agents in different localities. It may be favored by various protozoan or higher parasites accidentally present, and by digestive disturbances due to improper feeding.

To utilize animals infected in the natural way as a source of virus, is to put the experiments into nature's hands as far as possible without losing control of the main conditions. Such experiments cannot, therefore, be completely reproduced at will as is possible in the laboratory with pure cultures, and the results may vary from place to place. They are practical rather than scientific tests in the sense that the practical test may involve unknown factors largely eliminated in the scientific or laboratory test.



## NOTE ON COCCIDIA IN SPARROWS AND THEIR ASSUMED RELATION TO BLACKHEAD IN TURKEYS.

BY THEOBALD SMITH, M.D., AND ERNEST W. SMILLIE, D.V.M.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, January 4, 1917.)

The immediate inducements to follow coccidiosis in sparrows were their constant appearance in the unprotected yards of the incubator turkeys described in the preceding article and the view of Hadley<sup>1</sup> that the causal parasite described by one of us in 1894<sup>2</sup> as *Amæba meleagridis* is merely a stage in the development of the avian coccidium *Eimeria avium* and is disseminated and transmitted by various species of birds. Although no proof of this relationship has been furnished and the evidence accumulated is wholly against the hypothesis, one of us has not lost any opportunity to keep it in the foreground for the time being. Coccidiosis is widespread and when the invasion is intense and associated with the blackhead parasite, confusion may arise.

In all, 54 sparrows were trapped or shot while in the turkey yard. No lesions suggesting acute or chronic disease were found in any of them. The search for coccidia was carried on by the examination of fresh scrapings of the duodenal mucosa for the sexual stages and the rectal contents for the oocysts. In addition to this routine examination, cultures were made of the rectal contents of a certain number, to stimulate spore formation, as follows: 2 per cent agar-agar was suspended in a 0.5 per cent aqueous solution of sodium chloride, boiled, cleared, tubed, and autoclaved. When needed, this medium was poured into Petri dishes and allowed to set. Parti-

<sup>1</sup>Cole, L. J., and Hadley, P. B., *Rhode Island Agricultural Experiment Station, Bull. 141*, 1910.

<sup>2</sup>Smith, T., *U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. 8*, 1895, 7.



cles of feces were smeared over the surface of the agar and the dishes placed in a moist chamber in the incubator at 37°C. and at room temperature.

From a number of sparrows, portions of the duodenum were fixed in Zenker's fluid and alcohol and sectioned for study.

Table I summarizes the observations made.

TABLE I.

Sparrow No.	Sex.	Date.	Duodenum.	Rectal contents.
		<i>1916</i>		
1	Male.	July 12	+++	Not examined.
2	"	" 14	++	" "
3	Female.	Aug. 5	+++	" "
4	?	" 5	+++	" "
5	Male.	" 7	+	" "
6	Female.	" 7	+	" "
7	"	" 9	++	+
8	?	" 10	+	-
9	Female.	" 10	-	++
10	"	" 11	+	+
11	?	" 13	+	-
12	Female.	" 15	+	-
13	Male.	" 15	++	-
14	"	" 15	-	-
15	Female.	" 16	+	-
16	"	" 18	+	-
17	Male.	" 21	++	-
18	Female.	" 23	+++	++
19	"	" 24	+++	+++
20	"	" 28	+++	+
21	"	Sept. 1	++	+
22	Male.	" 3	+	+
23	Female.	" 7	+++	+
24	"	" 8	+++	++
25	"	" 11	+	+
26	"	" 15	+++	+++
27	"	" 15	+++	+++
28	"	" 21	+++	+++
29	"	" 21	+	+
30	Male.	Oct. 1	-	-
31	Female.	" 2	+	+
32	"	" 2	+++	+++
33	"	" 2	+++	+++

TABLE I—*Continued.*

Sparrow No.	Sex.	Date.	Duodenum.	Rectal contents.
<i>1916</i>				
34	Female.	Oct. 5	—	+
35	"	" 11	++	++
36	"	" 19	+++	+++
37	Male.	" 20	+	+
38	"	" 22	++	+
39	Female.	Nov. 6	+	—
40	Male.	" 9	++	++
41	"	" 9	+	—
42	Female.	" 13	—	—
43	"	" 14	+	—
44	Male.	" 14	+	+
45	"	" 14	—	—
46	"	" 22	—	—
47	"	" 23	+	—
48	Female.	" 23	—	—
49	"	" 23	++	++
50	"	" 28	—	—
51	"	" 28	—	—
52	Male.	Dec. 19	—	—
53	Female.	" 19	—	—
54	"	" 30	—	—

The examinations began on July 12, 1916, and extended to January, 1917. In all, 54 birds were examined and coccidia were found in 43, or 80 per cent. The first thirty-eight sparrows were from the University campus in Princeton, the rest were caught near the Department buildings about 3 miles east of Princeton. The sparrows were all adults weighing from 25 to 35 gm. We knew of no criteria for distinguishing sparrows less than 1 year old from older birds. The relative abundance of coccidia is indicated by the number of plus signs in the columns of the table, the absence of coccidia by the minus sign.

The examination of sections of the small intestine of sparrows, prepared as described, showed an invasion of variable intensity in the duodenum and little if any farther down. The parasites were all in the sexual phase, macrogametes and microgametocytes being the only forms present. The location of all the coccidia was in the

epithelial layer of the villi usually nearer the tip of the villi. The parasites were situated in the nuclear zone of the epithelial cells, close to the basement membrane but not in the core of the villus. Where the villi were twisted or flattened, the gametes appeared to be in the subepithelial tissue, but a careful focussing and consideration of the plane of the section did not confirm such a supposition.

The total absence of the vegetative or multiplicative cycle in the cases studied suggests two possibilities. Either the asexual phase of the cycle is eliminated or suppressed or else it goes on early, possibly while the birds are still in the nest. We have had no opportunity of examining nestlings to verify one or the other supposition.

The cultures of feces were a surprise in as far as they uniformly demonstrated a two-spored oocyst rather than the four-spored species which we had been led to expect from Hadley's observations. Each spore cyst contained four sporozoites. The development of the cyst contents proceeded quite rapidly and was complete within 3 days at room temperature or within 16 hours at 37°C.

It is of interest to note here that in one bird (No. 39) scrapings from the duodenum showed a matured oocyst with two spores, each containing developed sporozoites. This must have been taken in with the food and was detected before the sporozoites had escaped to begin the invasion.

Hadley<sup>3</sup> in a paper on coccidiosis in the English sparrow did not state what the infesting species was, but referred the reader to a later paper. In 1911,<sup>4</sup> he described *Eimeria avium*, a four-spored coccidium, and from the context of the paper the reader must assume that *Eimeria avium* is the sparrow parasite. The two-spored species is not referred to in the above papers and no cultures of the oocysts are mentioned. Some evidence that coccidia infesting turkeys and those infesting sparrows are different is presented by his measurements of the mature cysts which in the turkey average 14 micra by 21 micra, and in the sparrow 22.7 micra by 21.3 micra. The few measurements made by us of cysts from the rectal contents agree closely with his figures of the sparrow coccidium. Thus in two cases

<sup>3</sup> Hadley, P. B., *Centr. Bakteriolog., 1te Abt., Orig.*, 1910, lvi, 522.

<sup>4</sup> Hadley, *Arch. Protistenk.*, 1911, xxiii, 7.

the cysts were circular, measuring 18.7 micra and 17.5 micra respectively. In another case the axes were 18.7 micra by 22.1 micra. It is obvious that before drawing generalizations as to the transmission of coccidia from one species of host to another, we must first determine the character of the parasite regularly found in each host. For this determination the measurements of cysts are not very reliable.

The precise nature of the two-spored coccidium in the sparrow we did not attempt to determine. Two-spored coccidia in birds were found by Labbé,<sup>5</sup> who created the genus *Diplospora*. Sjöbring in 1897<sup>6</sup> describes two species in birds, one probably *Eimeria avium* and the other a two-spored species to which he gives the generic name *Isospora*. It is evident that even this superficial dipping into the literature proves the need for a thorough investigation, first of the coccidia of domesticated birds, and second of those of the wild species. It should furthermore be determined which birds are invaded by both species of coccidia and which harbor but one species.

The susceptibility of the incubator turkeys to the coccidia of the sparrows infesting their yards was not conspicuous. As a result of numerous examinations of intestinal scrapings and contents during the whole season, only two turkeys were found harboring coccidia cysts resembling those of the common species.<sup>7</sup> In No. 132, a small number of cysts were found in the cecal and rectal contents measuring 18 micra by 30.4 micra. In feces smeared on agar plates three cysts were subsequently found, two of which had developed each two spores with four sporozoites each. The third cyst remained undeveloped. In a second turkey (No. 155), one cyst was found in the cecum. In neither of these cases were coccidia found in sections of the duodenum, ileum, or ceca. The multiplication must have been very light or the ingested cysts may have passed through undeveloped.

<sup>5</sup> Labbé, A., *Compt. rend. Acad.*, 1893, cxvi, 1300.

<sup>6</sup> Sjöbring, N., *Centr. Bakteriöl., 1te Abt.*, 1897, xxii, 675.

<sup>7</sup> Smith, J. *Exp. Med.*, 1917, xxv, 405.

## SUMMARY.

Of 54 sparrows examined in or near Princeton, coccidia were found in 43, or 80 per cent. Most of the negative cases were encountered in November and December. In the summer and fall practically all were infected. These figures agree closely with Hadley's, who found 79 per cent infected from May to December.

Cultures of feces on agar showed that, at least in this locality, the infecting species belongs to the genus *Isospora* or *Diplospora* and not to *Eimeria*.

In a recent paper by Hadley,<sup>8</sup> which came into our hands after the manuscript had gone to press, the author now refers blackhead to invasions of *Trichomonas*. It is not possible to consider here the evidence on which this conclusion is based.

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<sup>8</sup> Hadley, *Rhode Island Agricultural Experiment Station, Bull. 166*, 1916.

## INTESTINAL OBSTRUCTION.

### AN EXPERIMENTAL STUDY OF THE INTOXICATION IN CLOSED INTESTINAL LOOPS.

By LESTER R. DRAGSTEDT, JAMES J. MOORHEAD, M.D., AND  
FRED W. BURCKY.

*(From the Hull Physiological Laboratory of the University of Chicago, Chicago.)*

(Received for publication, November 9, 1916.)

The cause of death in acute intestinal obstruction has not been determined. It is generally conceded, however, that a systemic bacterial invasion by the organisms from the obstructed intestine does not occur. As shown by McKenna (1), a bacteremia is only rarely found in clinical cases of death from acute intestinal obstruction. The absence of a systemic bacterial invasion in experimental obstruction has been demonstrated by a number of workers. The theory of a nervous disorder, as a result of anemia of the medullary centers following the loss of fluids in ileus, has received no experimental support. Both the clinical and experimental evidence so far obtained points to a quickly developing and rapidly fatal toxemia.

Hartwell, Hoguet, and Beekman (2) conclude from experiments on dogs that death in experimental intestinal obstruction is due, first, to a dehydration or loss of water from the tissues as a result of the excessive drain into the lumen of the intestine and vomiting, and, second, to the presence of a toxin in the circulating blood. The toxin is believed to be normally present in the lumen of the intestine, and the factor admitting this toxin into the blood is the damage to the intestinal mucosa. The life of the animal after experimental obstruction may be prolonged by the subcutaneous injection of normal saline solution.

Murphy and Brooks (3) conclude from their experiments on dogs with experimental intestinal obstruction, that death is due to toxemia, independent of infection of the peritoneal cavity or the general circulation. The toxin is formed as a result of bacterial growth in the obstructed intestine. It is not absorbed through a normal mucosa, but gains entrance to the blood stream when the mucosa of the intestine has been damaged. They emphasize the significance of the abnormal absorption in the production of the symptoms.

Whipple, Stone, and Bernheim (4) believe that death in these cases of obstruction is due to the absorption directly into the blood of a perverted secretion



of the duodenal or upper jejunal mucosa. They assume a disturbed physiological balance of the mucosa by which abnormal products are formed and secreted into the blood stream. This perverted secretion is a highly toxic substance and they claim that it can be recovered from the lumen of the intestine in cases of obstruction. Some work has been reported on the chemical nature of this toxic substance and attempts have been made to immunize dogs to it.

Draper (5) believes that the cause of death in intestinal obstruction is due to an aberrant activity of the duodenal and probably the pancreatic cells. He appears to have forsaken the view taken by him several years ago of a normal toxic secretion from the duodenal mucosa which is neutralized by the secretions of the intestine lower down.

There has not been a sufficiently thorough investigation of the chemical nature of the toxic substances found in the lumen of the intestine in cases of intestinal obstruction to determine whether they are essentially different from the toxic substances shown by Roger and Garnier (6) to be present in the lumen of the normal stomach and intestine. We should expect to find materials, in such a mixture of food substances and bacterial end-products as exists in the normal alimentary tract, which are toxic when injected into the blood stream. That the substances from the intestine in cases of obstruction are apparently more toxic than the normal contents of the intestine is not surprising. The accumulation of food products and secretions and the unrestricted activity of the intestinal bacteria might easily explain the increase in toxicity. Moreover, an intravenous injection of substances from an obstructed intestine producing toxic symptoms does not indicate that such a procedure occurs in cases of acute obstruction in which these toxic materials are separated from the blood by a layer of living cells. It is the function of the cells to change the substances in the lumen of the intestine to substances which can enter the blood stream without harm to the organism. The normal physiological function of the cells of the intestinal mucosa is both secretory and absorptive. Any pathological alteration (anemia through disturbance to the blood supply, traumatism, etc.) or intoxication of these glandular structures would, we should expect, lead to a depression and not a stimulation of their activity. We should expect a decrease and not a sudden increase in the absorptive properties of these cells under conditions such as exist in intestinal obstruction. Indeed, it has been shown by Braun

and Boruttau (7) and others that there is in reality a decrease in absorption from the intestinal lumen in cases of acute experimental obstruction. If, as maintained by some of the workers, the toxemia of obstruction is due to the absorption of toxic substances normally present in the intestine, made possible through the injury to the intestinal mucosa, this toxemia should be manifest in other inflammatory conditions of the intestine such as are present in ulcer, typhoid fever, amebic dysentery, etc., where there is extensive destruction of the mucosa.

The theory of a non-bacterial or physiological formation of the toxic substances found in the intestine in cases of acute obstruction, as advanced by Whipple and his associates and in a modified form by Draper, is not conclusive. The theory that under conditions of obstruction the normal physiology of the mucosa cells is so changed that they secrete a powerful toxin into the circulation is not probable. Especially is this so since cases of congenital atresia of the duodenum and other parts of the intestine are not rare. The infant shows no signs of toxemia or untoward symptoms until bacteria get into the alimentary tract after birth. The secretions of the intestinal glands begin early in intra-uterine life, and if there is anything significant in obstruction alone in stimulating a toxic secretion, such a secretion and toxemia would be manifest before birth. This does not exclude the factor of bacteria and food coupled with the anatomical condition of obstruction as important in the stimulation to a perverted secretion. From the work of others and from experiments to be reported here, this explanation seems unlikely.

To summarize briefly, death in acute intestinal obstruction is held to be due to a toxemia. That there are toxic substances formed in the intestine is established. That the substances found in the lumen of the intestine in obstruction are more toxic than the substances normally present is indicated. The presence of toxic substances in the blood in obstruction has not been shown. The relation of the toxic substances in the intestine in experimental obstruction to the symptoms, and the factors involved in the production and absorption of these toxic substances are the fundamental questions in the problem of intestinal obstruction which remain unsettled.

All the experiments were done under complete ether anesthesia and with careful aseptic technique. In no case was there an extensive wound infection, and in no instance was there a peritonitis from the operation itself.

*Isolated Closed Loops of the First Part of the Jejunum.*

Experiments were performed on five dogs, but the results were so uniform that only one protocol will be given.

*Dog 1.*—May 12, 1916. An isolated closed loop, about 12 cm. in length, of the first part of the jejunum (Text-fig. 1) was made, the continuity of the intestine reestablished by end to end suture, and the ends of the isolated loop were carefully infolded and closed. Care was taken not to injure the blood supply to the isolated loop.

May 13. Dog recovered from the immediate effects of the operation; appeared lively.

May 14. Dog weak; vomited on drinking water.

May 15. Found dead and cold in the cage.

*Autopsy.*—Performed at once. There was a considerable amount of fluid in the abdominal cavity, and the peritoneum was markedly injected, with evidence of a general peritonitis. The loop was greatly distended and perforated in the center, opposite the mesentery. Aside from the region of the perforation, the mucosa was practically normal. Microscopic examination showed merely a slight round cell proliferation and desquamation of some of the surface cells. The lungs, liver, and kidneys showed no change. There was a slight cloudy swelling in the spleen.

All the dogs in this series died within 96 hours and all showed a perforation of the closed loop and a general peritonitis. The perforation was evidently primarily caused by the accumulation of fluids in the closed loop. The distention was probably sufficient to shut off the blood supply to a portion of the mucosa which, becoming necrotic, rapidly gave way. We believe that death in these cases is due to the perforation and the sudden liberation of bacteria and their toxic end-products into the abdominal cavity where they are rapidly absorbed. The effects of the perforation cannot be essentially different from those in perforated ulcers and there seems to be no reason to believe that the small area of necrosis in these cases plus bacteria could give rise to a sufficient amount of toxic substances rapidly

enough to produce the symptoms observed. The inflammatory condition of the intestine adjacent to the perforation is not more marked than that found in any acute enteritis.

### *Isolated Closed Loops of the Lower Intestine.*

*Dog 2.*—Apr. 6, 1916. An isolated loop, 12 cm. in length, of the lower part of the jejunum was made, the intestinal canal reestablished by end to end suture, and the ends of the isolated loop were carefully infolded and closed. The blood supply to the loop was not damaged.

Apr. 7. Dog lively.

Apr. 9. Dog appeared toxic; vomited on drinking water.

Apr. 10. Found dead and cold in cage.

*Autopsy.*—Performed at once. The abdominal organs were normal except for the loop which was markedly distended and perforated causing a general peritonitis.

*Dog 3.*—Apr. 20, 1916. An isolated loop of the lower end of the ileum about 12 cm. in length was made, the intestinal canal reestablished by end to end suture, and the ends of the isolated loop were carefully infolded and closed. The blood supply to the loop was not damaged.

Apr. 21. Dog lively.

Apr. 25. Dog found dead and cold in cage.

*Autopsy.*—Performed at once. The closed loop was greatly distended and perforated causing a general peritonitis.

*Dog 4.*—May 25, 1916. An isolated loop, 12 cm. in length, of the transverse colon was made, the intestinal canal reestablished by end to end suture, and the ends of the isolated loop were carefully infolded and closed. The blood supply to the loop was not damaged.

May 26. Dog lively.

May 28. Dog able to eat and drink; appeared normal.

June 4. Dog died of distemper.

*Autopsy.*—Perforation of the closed loop which had been sealed by the omentum. There was a localized collection of pus about the perforation, but no general peritonitis.

In two cases isolated closed loops of the intestine in the region of the lower jejunum were made, the dogs living 4 and 5 days respectively. Death in each instance was caused by a perforation of the closed loop and the liberation of the toxic substances into the abdominal cavity. The dog with the closed loop of the ileum lived 5 days, death being due to the perforation peritonitis. Dogs with closed loops of the colon may live for weeks with no pathological symptoms.

*Isolated Closed Loops of the Duodenum.*

*Dog 5.*—July 6, 1916. An isolated loop of the duodenum, about 12 cm. in length, below the lower pancreatic duct was made, the continuity of the duodenum reestablished by end to end suture, and the ends of the isolated loop were carefully infolded and closed, great care being taken not to injure the large duodenal vein which at this point runs parallel to the intestine.

July 7. Dog highly toxic; refused water; lay quietly in corner of cage.

July 8. Dog died 48 hours after the operation.

*Autopsy.*—Performed at once. No peritonitis. The abdominal organs were normal except the closed loop. The loop was markedly distended and gangrenous but not perforated. The fluid in the loop was of the consistency of thick soup, bloody, and very offensive. It contained great numbers of bacilli and cocci. The mucosa of the loop was necrotic, coming away from the wall of the intestine as a brick red powder.

Six dogs were operated upon as described above and all died within 50 hours. At autopsy there was a perforation of the loop and general peritonitis in two animals, but in the remaining four the loops were distended but intact. In every instance the loop was black and gangrenous as compared with the remainder of the intestine. The mucosa always showed the same change and was markedly necrotic. The lungs, liver, and spleen were practically normal.

*Closed Pavlov Pouch.*

*Dog 6.*—Feb. 29, 1916. An operation for making a regular Pavlov pouch was performed, but instead of making a fistula, the small stomach was entirely closed (Text-fig. 2).

Mar. 1. Dog lively; no signs of toxemia.

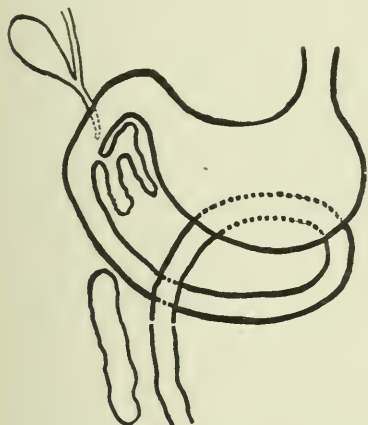
Mar. 2. Dog died 54 hours after the operation.

*Autopsy.*—Performed at once. The small stomach or pouch was greatly distended and perforated opposite the sutures. General peritonitis. The mucosa of the stomach and duodenum was not injected.

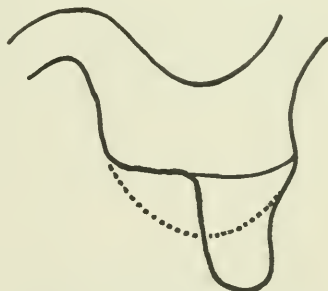
The experiments described in the above four groups are in part a duplication of experiments reported by other workers. Our purpose was to corroborate their findings and to establish a basis for our later experiments. They bring out the following facts: (1) Dogs with short closed isolated loops of the intestine die, those with loops in the upper part more rapidly than those with loops lower down. (2) There is no excessive vomiting of fluids with a resultant dehydration



of body tissues. (3) Dogs with closed isolated loops of the jejunum and ileum always show a perforation of the loop and general peritonitis at autopsy. Aside from the perforation the mucosa is not markedly changed. (4) Dogs with closed isolated loops of the duodenum die more quickly than dogs with closed loops in any other part of the intestine. At autopsy there may be an intact closed loop and no signs of peritonitis. In all cases where death occurs rapidly (within 48 hours), the mucosa of the closed loops is necrotic. In these cases the loops are distended with a fluid which contains great numbers of putrefactive bacteria.



TEXT-FIG. 1. Isolated closed loop of the first part of the jejunum.



TEXT-FIG. 2. Closed Pavlov pouch.

*Isolated Closed Loops of the Upper Part of the Jejunum, Previously Washed with Sterile Water and Ether.*

Isolated loops of the jejunum just below the duodenojejunal flexure were made (Text-fig. 1) and the continuity of the intestine was re-established by end to end suture. The isolated loops were then carefully washed with about 2 liters of sterile water and then with 2 liters of ether. A spray was used for this work and the fluids were introduced at sufficient pressure to distend the loops, thus affording a thorough washing of the mucosa. Following the washing, the ends of the loop were carefully wiped, infolded, and closed. Twenty-



five dogs were operated on in this manner and the postoperative course varied greatly. Sixteen of the dogs died after an interval varying from 2 to 6 days. In all the sixteen dogs there was a perforation of the loop with a resulting general peritonitis. That there had been a great distention of the closed loop before perforation was shown by the great thinness of the wall in places. The course in these dogs was no different from that in dogs in which the loops were not washed. The remaining nine dogs, which were operated on as above, and in which the isolated loops were first carefully washed with sterile water and ether, quickly recovered from the immediate effects of the operation and never showed any subsequent ill effects. Some of the dogs were later operated on and the loops examined. Others were not interfered with following the operation. They are still alive (5 months) and will probably continue to live indefinitely. Dogs which were opened from 2 to 3 weeks following the operation showed a fairly uniform picture. In nearly all, the ends of the loop remained tightly closed and aside from a slight distention the loops appeared practically normal. The fluid in the loops was gray in color and of the consistency of thick soup. Bacteriological examination showed the presence of *Bacillus coli* and a small coccus in most instances.

Merely washing the isolated loops with sterile water before closure does not suffice to save the animal from death within a week, although it prolongs life somewhat. There are at least four explanations of the absence of symptoms and death in the case of the ether washing of the loop. (1) The specific bacterium causing the elaboration of the toxic product in the closed loop may have been removed. The bactericidal property of the ether itself may have been sufficient to destroy the bacteria, or the hyperemia incited by the ether as an irritant may have been instrumental in aiding phagocytosis. (2) The substrate upon which bacteria act to produce the toxic product may have been washed out by the ether. (3) The secretory activity of the cells may have been sufficiently depressed to prevent the accumulation of enough fluids to distend the intestine to the point of rupture. The recovery of the cells from the effects of the anesthetic was probably sufficiently gradual to permit the establishment of an equilibrium between the amount of secretion into the lumen of the in-

testine and the amount of absorption. (4) The absorptive properties of the cells of the washed loops may have been increased through the removal of the lipid envelopes of the cells by the ether.

*Isolated Closed Loop of the Jejunum, Previously Washed with Sterile Water and Ether.*

*Dog 7.*—June 29, 1916. An isolated loop of the first part of the jejunum about 12 cm. in length was made, carefully washed with 2 liters of sterile water and 2 liters of ether, the ends were infolded and closed, and the continuity of the intestine was reestablished by end to end suture.

June 30. Dog lively.

July 1. No signs of toxemia.

July 4. Dog able to eat and drink; appeared normal.

July 8. Laparotomy and examination of the loop. The ends of the loop remained tightly closed. There was a large perforation 2 by 4 cm. in the middle of the loop opposite the mesentery. No evidence of peritonitis. The peritoneum was smooth and shining. There were a few adhesions about the end to end joint in the intestine. The perforation was closed by double rows of Lembert sutures and the loop dropped back into the abdomen. The blood supply to the returned loop was good.

July 10. Dog weak; lay in corner of the cage.

July 12. Dog well and lively.

July 20. Dog apparently in good condition; etherized.

*Autopsy.*—The abdominal cavity was normal except for the loop, which was entirely surrounded by omentum. It was tightly closed and moderately distended. The fluid in the loop was thick and yellow in color. The mucosa of the loop was normal on gross and microscopic examination. Cultures taken from the loop fluid remained sterile.

The subsequent perforation of the isolated closed loop, in this experiment, without symptoms of intoxication or evidence of peritonitis indicates that the fluids responsible for the distention of the loop and consequent perforation are not necessarily formed as a result of bacterial activity. They are probably non-toxic secretions from the intestinal cells, the activity of which could not have been permanently inhibited by the ether. The lumen of the intestine must have been practically sterilized by the washings or else the perforation must have occurred before there was any marked proliferation of the bacteria and elaboration of their toxic end-products. In this instance the formation of the closed loop did not suffice so to alter the normal

function of the jejunal cells as to stimulate them to the formation of a toxic secretion, either into the blood stream or into the lumen of the intestine. The perforation of the loop allowed free access of the bactericidal abdominal juices into the lumen of the isolated loop and consequent sterilization, as shown by the negative cultures taken on July 20. This experiment demonstrates that an aseptic isolated closed loop of the upper jejunum is not incompatible with life.

*Isolated Closed Aseptic Loop of the Lower Jejunum, in Which the Blood Supply Was Occluded with No Untoward Symptoms.*

Dog 8.—Apr. 19, 1916. An isolated closed loop of the lower part of the jejunum about 12 cm. in length was made, having been previously washed with sterile water and ether, the ends were infolded and closed, and the continuity of the intestine was reestablished by end to end suture. The blood supply to the loop was not damaged.

Apr. 21. Dog made a good recovery; no toxic symptoms.

July 3. Dog in good condition; laparotomy and examination of the loop. The isolated loop was open at one end, and the secretions of the loop had free drainage into the abdominal cavity. The loop was normal in appearance. There was no evidence of peritonitis, although a few cheesy white flakes could be found in the mesentery. The end of the loop was closed and the entire blood supply to the loop was occluded by double ligatures around the vessels in the mesentery. Cultures taken of the contents of the loop before closure remained sterile.

July 5. Dog recovered from the operation and showed no toxic symptoms.

Oct. 10. Dog was in good condition and had never shown any ill effects from the operation. Laparotomy and examination of the abdominal contents were done. The intestinal loop had entirely disappeared and the only trace of it that could be found was a bit of fibrous connective tissue in the mesentery in the region of the end to end joint in the intestine.

This experiment confirms the results obtained with Dog 7. The washings probably reduced the bacterial content of the intestine to such an extent that the bactericidal juices of the abdomen were able to cope with the remaining bacteria. It shows that isolated intestinal loops of the lower jejunum can be effectually sterilized by this method and also that isolated closed aseptic loops in the region of the lower jejunum are not incompatible with life. It also establishes the fact that anemic necrosis of the intestine following occlusion of the blood supply produces no toxic symptoms when bacteria are excluded.

There is thus a simple aseptic infarct of the intestine. Evidently then the toxemia of intestinal obstruction cannot be due to circulatory changes in the intestine in the absence of bacteria.

*Isolated Closed Loop of the Jejunum. Recovery. Death on Subsequent Occlusion of the Blood Supply.*

*Dog 9.*—June 30, 1916. An isolated loop of the first part of the jejunum about 12 cm. in length was made, washed with sterile water and ether, the ends were infolded and closed, and the continuity of the intestine was reestablished by end to end suture.

July 2. Dog recovered from operation; showed no toxic symptoms.

July 10. Dog in perfect health; laparotomy and examination of the loop. There were no signs of peritonitis. The loop was markedly distended but not perforated. The walls of the loop were normal in appearance, thickness, and color, and the blood supply was good. The blood supply was entirely occluded by double ligatures about the vessels in the mesentery and the loop dropped back into the abdomen.

July 11. 24 hours after the operation the dog was found dead.

*Autopsy.*—Performed at once. The abdomen was distended with gas and bloody, offensive fluid. The loop was entirely disintegrated and separated from the mesenteric stump. Cultures taken from the abdominal cavity showed *B. coli*.

It will be noted that washing the isolated loops of the intestine does not in itself completely remove all the organisms present. It was the subsequent entrance of the bactericidal abdominal juices in the ruptured loops that completed the sterilization in these cases. *Bacillus coli* and cocci were found in the closed loops that were later examined. In this experiment, then, there were probably a great number of bacteria and their end-products in the lumen of the loop. It has been shown that bacteria proliferate rapidly in these closed loops where the conditions for their growth are favorable. However, the mucosa of the intestine is able to prevent the entrance of bacteria or their toxic products into the blood in sufficient amounts to produce symptoms. This is a normal function of the mucosa of the intestine and is evidently not changed by the condition present in a closed isolated loop. The secretion into the lumen of the loop is practically balanced by the absorption from the intestine so that whatever distension occurs is gradual enough for the blood supply to accommodate itself. Thus a condition of anemia and necrosis of



any part of the loop is prevented. However, when the entire blood supply was suddenly occluded, the loop became at once necrotic, and this necrotic tissue plus the enclosed intestinal bacteria resulted in the formation of toxic products, which when liberated into the abdominal cavity and absorbed caused death. Whether the mere liberation of the contents of the loop without necrosis of the tissue would have caused death we do not know. However, from the fact that the liberation of the contents from a washed loop caused no symptoms, it would seem that this tissue necrosis is an important factor.

*Isolated Closed Loops of the Lower Duodenum, Previously Washed with Sterile Water and Ether.*

Seven dogs were operated on as in Text-fig. 3, the isolated loop of the lower duodenum was carefully washed with sterile water and ether, and the ends were infolded and closed. All the dogs died within 72 hours. There was no marked difference in the post-operative course of these dogs and the dogs in which the closed loops of the duodenum had not been previously washed. All made an uneventful recovery from the immediate effects of the operation and showed nothing abnormal for the first 12 hours. Then symptoms of toxemia, extreme muscular weakness, vomiting, diarrhea, and muscular tremors, appeared, followed by prostration and death. At autopsy the isolated loops were greatly distended and black or covered with purple blotches. In four animals there was a perforation with signs of a beginning general peritonitis. In three, however, there was no perforation and no signs of peritonitis were present. The loops were all distended and black in appearance. In every instance they were filled with a foul smelling bloody fluid. This fluid was found to contain large numbers of *Bacillus coli*, cocci, and other bacilli. In all the dogs the mucosa was necrotic and in some it appeared as a red granular powder.

There are two probable factors accounting for our failure to keep the dogs with the washed closed loops of the duodenum alive. In the first place the upper part of the intestine is mainly secretory and very slightly absorptive in function. Consequently there is little chance to establish an equilibrium between secretion into the intestine

and absorption from it. Second, the arrangement of the blood supply to the duodenum is different from that in any other part of the digestive tract. The vessels supplying the duodenum run parallel to the lumen of the intestine. Any slight distention of the duodenum may easily occlude these vessels and thus shut off the circulation to the entire duodenum. We attribute the failure to keep dogs with closed loops in this region alive as due to these two factors and not to anything peculiar in the quality of secretion of the glands in the duodenum or in the bacterial flora.

*Experiments to Determine the Part of Ether in the Washed Isolated Loops.*

*Dog 10.*—May 17, 1916. An isolated loop of the first part of the jejunum about 12 cm. in length was made, washed with sterile water, the ends were infolded and closed, the continuity of the jejunum was reestablished by end to end suture, and the loop filled with ether and dropped back into the abdominal cavity.

May 19. Dog found dead.

*Autopsy.*—Performed at once. There was a perforation of the closed loop, and signs of a beginning general peritonitis were present. The mucosa aside from the region of the perforation was normal.

Two dogs were operated on in this manner and both died within 48 hours. The experiment was undertaken to find out if the function of the ether was to remove a substrate upon which bacteria might act to produce a toxic product. If the ether was left in the loop, its germicidal and its anesthetic effects on the cells would be the same, but no substrate would be removed. However, the experiment is subject to the criticism that the ether enclosed in the loop when warmed to the temperature of the body might exert sufficient pressure to rupture the loop and liberate the contents.

*Isolated Closed Loops of the Jejunum and Colon, Previously Washed with Sterile Water and 70 Per Cent Alcohol.*

Closed loops of the upper part of the jejunum were made in seven dogs, the loops being carefully washed with sterile water and 70 per cent alcohol before closure. Of these seven dogs, five died within 4 days with perforation of the loop and general peritonitis. One died in 5 days with a peritonitis from a leak in the joint. The iso-



lated loop in this animal was distended but intact. Cultures taken from the loop fluid showed *Bacillus coli*, a streptococcus, and *Bacillus subtilis*. One of the dogs lived 18 days, but died with a peritonitis from perforation of the closed loop.

Isolated closed loops of the transverse colon, washed with sterile water and 70 per cent alcohol, were made in three dogs. One dog died after 3 days with a perforation of the loop and resulting general peritonitis. The other two showed no untoward symptoms and were later opened and the loops examined. In one of the dogs opened 19 days after the first operation, the closed loop was found intact but somewhat distended. The other dog was opened after 28 days and the loop removed. Here also the loop was distended and intact. In both these animals the fluid in the closed loops was of a dirty chocolate color, foul odor, and contained great numbers of bacteria.

It will be seen that dogs with alcohol-washed loops live longer than dogs with simple closed loops or dogs in which the loops have been washed with sterile water before closure. It is probable that alcohol acts in much the same manner as ether.

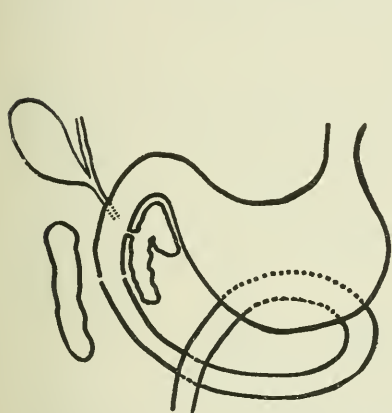
*Isolated Loops of the Upper Jejunum, Previously Washed with Sterile Water and Lysol Solution. Ends Closed.*

A 2 per cent solution of lysol was used and experiments were performed on two dogs. Both died within 48 hours with a perforation of the closed loop and a general peritonitis. In both the mucosa of the loop was necrotic and separated from the muscularis.

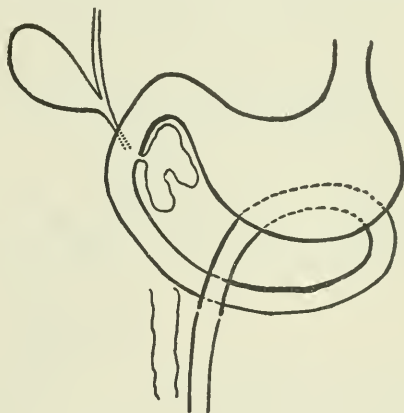
*Isolated Loops of the Jejunum, Previously Washed with Sterile Water, Dropped Back into the Abdomen with Both Ends Open.*

The perforation of closed loops of the jejunum and the non-appearance of peritonitis or toxic symptoms in some of the previous experiments, led us to attempt experiments of this kind. Four dogs were operated on as in Text-fig. 4, the isolated loops were made in the usual manner but were dropped back into the abdomen with both ends open. All the dogs made uneventful recoveries from the operation and never showed any toxic symptoms. Two of the dogs were

opened 3 weeks after the first operation and the loops examined. The ends remained open although the loops were surrounded by numerous adhesions. There was some light yellow fluid in the abdominal cavity and a few white cheesy flakes in the mesentery. The other two dogs were opened 4 and 5 weeks respectively after the first operation. The loops were buried in adhesions and both ends tightly closed. They were moderately distended. Pressure on the loops



TEXT-FIG. 3. Closed loop of the duodenum.



TEXT-FIG. 4. Open loop of the first part of the jejunum.

caused no escape of fluid from the sealed ends. The fluid in these loops was yellow and quite thick. Cultures on physiological blood agar were negative.

*Isolated Loops of the Duodenum Dropped Back into the Abdominal Cavity with Both Ends Open.*

*Dog 11.*—July 20, 1916. An isolated loop of the duodenum about 10 cm. in length below the lower pancreatic duct (Text-fig. 5) was made, the continuity of the duodenum reestablished by end to end suture around the loop, and the loop dropped back into the abdomen without washing and with both ends open.

July 23. Dog recovered from operation; showed no signs of toxemia.

Aug. 10. Dog in perfect health; nutrition has not suffered since operation; laparotomy and examination of the loop. Both ends of the loop were tightly sealed by adhesions. The loop was distended, but pressure applied to it did

not cause any fluid to escape from the ends. It was dropped back and the abdomen closed.

Aug. 11. Dog recovered from the operation.

Aug. 15. Dog apparently in perfect health no signs of intoxication since operation.

Aug. 20. Dog etherized.

*Autopsy.*—Performed at once. There was some thin yellow fluid and a few flakes of fibrin were found in the abdominal cavity. The isolated loop was closed and wrapped in dense adhesions. Thick gray fluid was present in the lumen. The mucosa was normal on gross and microscopic examination. No cultures were made of the fluid in the loop.

Six dogs were operated on in the above manner. Three died within 5 days of a general peritonitis. The remaining three showed no



TEXT-FIG. 5. Open loop of the duodenum.

untoward symptoms whatever after the operation. One was opened after 16 days, the isolated loop inspected, and the ends were closed. At operation the loop appeared normal and the blood supply was good. Following the second operation the dog developed a hernia and was etherized 3 days later. The other two dogs were opened 20 and 30 days respectively after the first operation. In both these animals the loops were tightly closed by adhesions and were distended with a thick yellow fluid. Cultures made in one case were negative. In both instances the muscularis and mucosa were normal on gross and microscopic examination.

The experiments with the open loops of the jejunum and duodenum show conclusively that the secretions of the jejunum and duodenum are not toxic enough to induce symptoms in dogs when allowed to

flow into the abdominal cavity. Whether these secretions are toxic when injected into the blood stream, we do not know. All the normal stimuli to the secretion of the glands of the intestinal mucosa are not present, but the secretion goes on, and it is probable that this secretion is qualitatively the same in the isolated open loops as in the intact intestine. There has been no damage to the secreting cells either through injury to the cells themselves directly (trauma in washing) or to their blood supply. The experiments also show that the theory of Draper of a normal toxic secretion of the mucosa of the duodenum which is neutralized by the jejunal juices is untenable. The juices of the open loops of the duodenum are absorbed directly from the abdominal cavity and do not come in contact with the mucosa of the jejunum and ileum. Also there is no perverted secretion or aberrant activity on the part of the cells of the duodenal mucosa when the factor of the bacteria of the intestine is excluded and when the blood supply is not occluded by a too rapid distention of the loops.

#### DISCUSSION.

It has been demonstrated that death in experimental intestinal obstruction may occur in the absence of a systemic bacterial invasion or of peritonitis. It is probable that the symptoms and death are due to a rapidly developing toxemia, although toxic substances have not been demonstrated in the blood in cases of experimental obstruction. As there was no excessive vomiting of fluids in our experiments the theory of dehydration of the body tissues receives no support. We were able to keep alive animals with closed loops of the duodenum and upper jejunum where bacteria had been previously removed from the loop by free drainage into the abdominal cavity. Inasmuch as the mucosa of these loops was not injured in any way, the theory of a perverted secretion of the mucosa cells induced by the condition of obstruction present in closed duodenal loops, as advocated by Whipple, appears untenable, as is also the theory of Draper of an aberrant activity of the cells of the duodenum and the pancreas. The early theory of Draper that the toxemia in obstruction was due to the absorption of toxic secretions of the duodenum which were normally neutralized by the juices of the intestine lower

down, is disproved by the experiments with the open duodenal and jejunal loops in which these unneutralized secretions pass directly into the abdominal cavity and are absorbed, without the production of toxic symptoms.

We were also able to keep alive dogs with closed loops of the intestine (loops washed with sterile water and ether) containing bacteria, but in which the blood supply had not been damaged by distention. Thus, it is probable that bacteria alone or in connection with the contents of the lumen of the intestine, do not give rise to the toxemia of obstruction, when they are separated from the blood by the cells of the intestinal mucosa. However, a combination of these two factors (necrotic tissue plus putrefactive intestinal bacteria) rapidly gives rise to fatal toxemia. It is probable that the death in the experiments with the unwashed isolated intestinal loops is identical with that following gangrene of the intestine. The fatal toxins are the result of the action of the putrefactive organisms on necrotic tissue. That dogs with closed loops of the duodenum die sooner than dogs with closed loops lower down in the intestine may be due to the fact that the duodenal loop becomes entirely necrotic on distention by reason of the arrangement of the blood supply while in the cases of the loops of the remainder of the intestine only the tissue in the immediate vicinity of the perforation becomes necrotic. Death in the latter cases may be caused by the peritonitis resulting from the perforation. The area of necrosis or gangrene in the lower intestine is usually so small that it is probable that sufficient toxic products to account for the symptoms could not be formed.

Just how far the conditions present in closed isolated unwashed loops of the intestine resemble those found in clinical obstruction is problematic. We believe that the symptoms and pathology of these simple closed loops resemble cases of acute obstruction in man in which there is an accompanying occlusion of the blood supply to a part of the intestine (volvulus, strangulated hernia, etc.). The toxemia resulting from closed intestinal loops is not similar to the toxemia resulting simply from a delay in the passage of food in the intestine and in which there is no disturbance of blood supply or injury to the tissue.



## CONCLUSIONS.

1. Closed intestinal loops in which bacteria are first removed are compatible with life.

2. Closed intestinal loops in which bacteria are present but in which tissue necrosis is prevented, are compatible with life.

3. Closed aseptic intestinal loops in which the blood supply is completely occluded are compatible with life.

4. The normal secretions and bacterial products of the duodenum and jejunum are not sufficiently toxic to produce any symptoms when allowed to drain into the abdominal cavity.

5. Our results do not support the theory of Draper of a normal toxic secretion of the duodenal mucosa, neutralized by the jejunal mucosa, or the perverted secretion theory of Whipple.

6. Bacterial activity plus necrotic tissue, or the absorption of toxic products resulting from the action of putrefactive bacteria on necrotic tissue is the important factor in the rapid death in simple closed intestinal loops.

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# THE INFLUENCE OF CERTAIN ORGANIC SUBSTANCES ON THE GROWTH OF THE TUBERCLE BACILLUS.

By PAUL A. LEWIS, M.D.

(From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.)

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In attempting to treat experimental tuberculosis by well defined chemical compounds, it is important to know what relations exist between the tubercle bacillus and the chemicals, under various conditions of exposure. The present paper is intended as a contribution to our knowledge of these relations in as far as they can be studied in the culture flask.

## HISTORICAL.

The general conception that it might be profitable to determine the power of substances to inhibit the growth of cultures, selecting the more active for subsequent therapeutic experimentation, was formulated by Robert Koch. The idea was completely developed by Bechhold and Ehrlich. The work of Koch and Bechhold and Ehrlich agreed in showing that substances might be highly active in the test-tube and still have no demonstrable capacity to sterilize the diseased animal. Ehrlich, moreover, showed conclusively in the course of his chemotherapeutic studies that substances without activity in the test-tube may be very active against the parasites in the body of the animal. In fact, until recently there was no instance known of a well defined chemical compound having pronounced disinfectant action in the test-tube and exerting the same in demonstrable degree in the animal body.

Schiemann<sup>1</sup> has recently developed the fact that salvarsan is active in the test-tube against anthrax bacilli, the bacillus of swine erysipelas, and *B. mallei* in dilutions of 1 : 1,000,000 or greater. Serum seems not to inhibit this action, but rather to favor it in certain instances. Against experimental infections with these microorganisms the drug also has a certain value. Ethylhydrocuprein (optochin), perhaps the one determinate chemical compound which has up to the present time been markedly effective *in vivo* against a bacterial infec-

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<sup>1</sup> Schiemann, O., *Z. Immunitätsforsch., Orig.*, 1915-16, xxiv, 167.

tion (pneumococcus), has marked, and in some degree, at least, specific activities against the tubercle bacillus *in vitro*.

Disinfectants as studied in the test-tube fall into two classes. One class is characterized by the possession of a more or less uniform action against all bacteria. If the action of phenol and mercuric chloride on a variety of bacterial species is compared, it is found that the mercurial compound is by far the more active agent (extraneous albumin being excluded from the reaction). The resistance of bacterial species also varies, some being susceptible, others relatively resistant. But if a ratio is drawn between the activity of phenol and bichloride of mercury for any single species of bacteria, that ratio will be approached closely by similar figures made up on the basis of experiment with any other race or strain of microorganism. Such substances are general disinfectants; they are more or less universal protoplasmic poisons, and, as a corollary to this, are apt to be proportionately far more toxic to higher animals than they are for microparasitic life.

The other, and from the point of view of experimental therapeutics, the more important class of disinfectants, is characterized by a limited range of action. Certain bacterial species only are affected in a marked degree by the typical members of the group. This is the ideal conception and one which is being more nearly fulfilled by the continued examination of substances from this point of view. In no instance, perhaps, up to the present time has the specificity displayed by the bacteriolysins of the blood serum of immune animals been uncovered as an attribute of well defined chemical compounds, although this result must be postulated as ultimately attainable.

The literature of this type of disinfectant action, designated by Ehrlich as *halb spezifische*, or partially specific disinfection, is not extensive.

It was shown by Boer (1890)<sup>2</sup> in certain tables that malachite green and methyl violet act differently than a number of other disinfectant substances, showing a somewhat unusual degree of variation in their activity against certain bacteria. *B. anthracis* and *B. diphtheriæ* were about equally resistant to mineral acids,

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<sup>2</sup> Boer, O., in Behring's *Gesammelte Abhandlungen*, Leipsic, 1893, pt. i, 198.

the stronger alkaline substances, salts of certain heavy metals, and phenol. *B. anthracis* was the more susceptible to malachite green in the proportion of 12 to 4, and to methyl violet in the ratio of 7 to 1. Boer did not comment on the possible significance of this observation.

Von Drigalski and Conradi (1902)<sup>3</sup> based a method for the differential isolation of *B. typhosus* on what they called the "elective bactericidal action" of certain aniline dyes—malachite green, brilliant green, methylene blue, methyl violet, and Krystallviolett were mentioned by them as showing this property. They recommended the addition to the culture medium of Krystallviolett in certain concentrations, especially to restrain the growth of cocci commonly found in the dejecta. Loeffler (1903)<sup>4</sup> found that a preparation of malachite green containing dextrin, when added to agar plates in concentrations of 1:1,000 to 1:4,000 restrained the growth of *B. coli* more than that of *B. typhosus*. He worked out and recommended a method for the rapid isolation of the typhoid bacillus on this basis.

Bechhold and Ehrlich<sup>5</sup> and subsequently Bechhold<sup>6</sup> developed most fully the partially specific nature of the disinfectant action of certain substances. The papers of these authors cannot be abstracted with advantage. Concerning our immediate problem, Bechhold reports some observations made with the tubercle bacillus, the substances tested having no demonstrable action against this species. Subsequently in a general review of this work, Bechhold<sup>7</sup> makes the statement that monochloronaphthol is highly active against the tubercle bacillus and *B. pyocyaneus*, but much less active against staphylococcus. He gives no exact figures nor does he state which of the several possible monochloronaphthols he employed.

DeWitt<sup>8</sup> in a study of the power of a number of aniline dyes to restrain the growth of various bacteria, records<sup>9</sup> the development of different bacterial species on agar to which the dyes had been added in the single concentration of about 1:8,000 (2 drops of a 1 per cent solution in 10 cc. of agar). Tests at a single concentration are not, of course, designed to bring out any specific quality which substances may possess. DeWitt's tables do, however, give some indication of this action. In most instances *B. subtilis* and *B. tuberculosis* were equally affected. With Bismarck brown, eosin A, erythrosin, and neutral red, the tubercle bacillus gave a feeble growth or none at all, while *B. subtilis* grew vigorously. With eosin (bluish) the reverse was found. In general, the growth of the tubercle bacillus was inhibited more readily than that of any other organism tested.

<sup>3</sup> von Drigalski, and Conradi, H., *Z. Hyg. u. Infektionskrankh.*, 1902, xxxix, 283.

<sup>4</sup> Loeffler, *Deutsch. med. Woch.*, 1903, xxix, 286.

<sup>5</sup> Bechhold, H., and Ehrlich, P., *Z. physiol. Chem.*, 1906, xlvii, 173.

<sup>6</sup> Bechhold, H., *Z. Hyg. u. Infektionskrankh.*, 1909, lxiv, 113.

<sup>7</sup> Bechhold, Desinfektion, in Ehrlich's *Festschrift*, Jena, 1914, 505.

<sup>8</sup> DeWitt, L. M., *J. Infect. Dis.*, 1913, xii, 68.

<sup>9</sup> See Table V.

DeWitt's results are possibly less significant than they otherwise might be because the culture of the tubercle bacillus which she used was, as she states, of "questionable" authenticity. It was a rapidly growing culture producing abscesses in guinea pigs. She states that: "These abscesses are acute or at least develop much more rapidly than tuberculous glands, and other pigs in the same cage are infected." These characteristics are certainly not typical of the tubercle bacillus.

Our study as presented in this paper represents the beginning of a general survey of available organic chemicals with the view to determining which of them are able in some partially specific sense to restrain the growth of the tubercle bacillus. As will become apparent in the course of the paper, such an effort as this is subject to many limitations both as to method and material and the results can have at most a limited value. They are presented with the hope that they may form a useful guide to others interested in this field of work.

#### *Method.*

In general our method has been to make various dilutions in glycerol bouillon of the substances employed. The flasks containing these mixtures were inoculated with a surface growth of the tubercle bacillus, certain control flasks of the same bouillon being inoculated at the same time. When the growth on the control flasks completely covered the surface, the experiment was terminated by a comparison and record of the extent of growth throughout the series. The flasks were then inoculated with one drop of a 24 hour bouillon culture of *Bacillus typhosus* and a similar comparison was made on the following day of the degree of growth of this microorganism. Some comparisons were made with other bacteria to determine the general suitability of the typhoid bacillus as a standard. It would be impossible to cover a wide field of this nature without establishing some sort of routine procedure and we shall describe briefly the one that we have adopted after much preliminary experimentation.

*Preparation of Solutions.*—The preparation of the test fluids varies with the concentration desired. We use as the basis for our observations 20 cc. of glycerol bouillon (acidity 1.5 to 2 per cent phenolphthalein) in Jena glass boiling flasks of 50 cc. capacity. This gives a comparatively uniform surface area to be covered. For con-



centrations stronger than 1 : 1,000 the substance to be tested is weighed directly into the empty flask. We use a torsion balance for this work. The balance is simple and rapid in its operation and is adjusted to a sensitivity of 1 mg. Weighing 20 mg. and adding 20 cc. of bouillon gives a concentration of 1 : 1,000. The possible error at this concentration is 5 per cent, the probable error is less than this and would be equalized in a series of tests. The flasks are conveniently labeled by a ring of paper cut to hang over the neck.

When a series has been weighed off, the flasks plugged with cotton are sterilized with dry heat. Most of the substances we have studied so far stand a temperature of 130°C. without decomposition when dry. This temperature continued for 3 hours will usually render the preparation sterile. The bouillon measured into similar flasks is separately sterilized in the autoclave. When ready it is poured into the flask with the weighed, sterilized substance. This can be accomplished with only small losses due to contamination. For lesser concentrations, 1 : 1,000 to 1 : 10,000, the 1 : 1,000 solution in bouillon is diluted with glycerol bouillon with suitable pipettes.

For concentration below 1 : 10,000 we start with 25 mg. of the substance sterilized by dry heat in a suitable flask and then add sterile water to make a 1 : 1,000 solution. This is then measured into the bouillon flasks; 2 cc. for 1 : 10,000, 1 cc. for 1 : 20,000, etc.

According to the method of preparation, it will be noted that the concentrations given in the tables are only approximately correct. The actual concentrations, 1 : 11,000, 1 : 21,000, 1 : 40,500, etc., can be substituted, but the round numbers are more convenient to handle and are close enough for our present purpose.

Many substances can be more simply handled by sterilizing the finished solution in bouillon. So large a number are either decomposed or enter into fixed combinations with bouillon constituents when treated in this way that we recommend the above procedure as a rule. Certain substances which can be sterilized successfully in watery solution decompose at the temperature mentioned when dry. They are relatively few and must be handled by other procedures.

*Cultures Used.*—As the culture for all this work we have used a strain of *Bacillus tuberculosis* obtained some years ago from The



Rockefeller Institute for Medical Research designated Bovine III. This culture is probably a transplant of the Bovine III of Theobald Smith. A comparison with the latter strain, however, shows that our culture grows rather more vigorously and is definitely less virulent for guinea pigs. Our culture does, however, give the characteristic disease in guinea pigs and rabbits, is acid-fast, and produces no acid in glycerol bouillon. It is evidently a culture of bovine type of low virulence. The results obtained with this culture have been checked in certain instances with other cultures of higher virulence, also vigorous growers, and found to be identical. We have so far made no comparison with recently isolated cultures, which offer an important problem, but a distinct one.

The stock culture for this work is kept on glycerol bouillon. Cultures about 3 weeks old have usually been used for the inoculation. The inoculation is made with a fine straight wire, a bit of the growth 1 or 2 mm. in diameter being carried over. For each separate lot of culture medium used, at least five control flasks have been inoculated in series with those test substances in which that particular bouillon was concerned. This is essential.

The culture Bovine III can be depended on to give a growth over all of the surface of flasks as we have used them in from 1 to 2 weeks. When the surface of all the control flasks is just overgrown we have terminated the observation period.

For the tests with *Bacillus typhosus* we have used the Rawlings strain obtained some time ago through the courtesy of Major Russell, of the United States Army.

*Recording Results.*—The results are recorded by an inspection of the flasks. The original bit of culture at least must remain visible if the experiment is to be considered conclusive. If it is shaken down by accident or sinks spontaneously, growth cannot occur from the bottom. The designations used are as follows:

No growth indicates that the original bit is thin and dry; very slight, that the original bit is thickened but shows no new surrounding film; slight, that there is a definite film of new growth extending from the original, the whole mass being 1 cm. or less in diameter; moderate, that the surface is about one-fourth covered; good, that the surface is one-fourth to three-fourths covered; and full, that the surface is completely covered or nearly so.

Occasional lots of bouillon are encountered in which after a period of growth there is a cessation with the surface of the control flasks about three-fourths covered. Tests in which this occurs must be discarded.

The results of the tests with *Bacillus typhosus* are recorded in the same terms based on the examination of a hanging drop taken from the flasks at the end of a 24 hour period of growth. The slight and moderate grade degrees of proliferation are frequently marked by phenomena of agglutination and involution which help to fix the amount of inhibition which prevails. A numerical expression could be obtained by plating, but it is doubtful if this would be of use for the present purpose in view of the fact that no such system could be applied to the tubercle bacillus.

*Materials Used.*—The substances reported in this paper are nearly all aniline dyes. They have been obtained partly by purchase in the market and partly by donation.<sup>10</sup> These commercial dyes contain a varying proportion of impurities, especially inorganic salts. They should in many instances undoubtedly be considered as mixtures of more or less closely related substances rather than as chemical entities. The salts should be considered as diluents. In the concentrations we are considering they probably have no effect on growth. In so far as it is possible that other impurities may exist in any preparation and increase its activity, the results now reported cannot be considered as final. On the whole, this is probably not a factor in many instances. To exclude it as a possibility in any case involves so much careful work that it can only be attempted on a selected material and as a special problem. We have already approached this matter in certain cases which seemed to be especially significant in their bearing on the influence of chemical constitution on the result. Such observations as we have made will be reserved for later publication.

<sup>10</sup> We are indebted to the American agents of Kalle and Company, and the Bayer Company, for sample lines of the azo-dyes put in the trade by their respective firms. We are also indebted to Professor W. T. Taggart of the University of Pennsylvania for large contributions from a collection of dyes maintained by the Department of Organic Chemistry. The dyes purchased have been obtained chiefly from Grüber and Kahlbaum.

## RESULTS.

The results of this survey as carried out up to the present time are given in Tables I to VI. The tables show the name of the preparation tested and its source. They show the greatest concentration at which growth is full for each culture of *Bacillus tuberculosis* and *Bacillus typhosus*. These concentrations are combined in a factor which is an expression of the relative susceptibility to the inhibiting action of the dye. The point of full growth was taken as the basis of this comparison because it permits of the consideration of a much larger number of substances than does the point of complete inhibition. The point of no growth for the typhoid bacillus can be determined in comparatively few instances. In certain cases it has been possible to compare the influence of substance for all concentrations from no growth to full growth with each bacterial species. In these cases the factor has been much the same with whichever concentration was made the basis for comparison.

The substances have been arranged in the tables in groups according to their activity against the tubercle bacillus. With the substances showing much activity it is usually possible to show a restraining action on the typhoid bacillus at some concentration. For less active substances the typhoid organism frequently grows without restraint in a saturated solution. In these cases it is only possible to indicate that the factor is greater than the value actually determinable.

The interest of the laboratory has centered for some time on the action of certain azo-dyes which are vital stains. For this reason the number of this class of dyes examined is large, and out of proportion to those of other constitution. These are tabulated by themselves in the case of the more active ones. In making this separation we have relied in most instances on the tables of Schultz.<sup>11</sup>

*Interpretation of Results.*—There is perhaps little that can be said at this time in extension of the actual results of the work presented. It is evident that a great variety of substances have marked capacity to restrain the growth of the tubercle bacillus. Those dyes whose disinfectant activities have long been known to be considerable,

<sup>11</sup> Schultz, G., Farbstofftabellen, Berlin, 5th edition, 1914.

TABLE I.

*Dyes of Various Groups Strongly Restraining Growth of the Tubercle Bacillus.*

Designation.	Source.	Tubercle bacillus growth full at.	Typhoid bacillus growth full at.	Typhoid bacillus Tubercle bacillus
Acridinorange .....	G.*	1 : 1,000,000	1 : 4,000	$\frac{250}{1}$
Äthylrot.....	Kahl.	1 : 200,000	1 : 1,000 (Saturated.)	$\frac{200}{1} +$
Auramin.....	G.	1 : 200,000	1 : 4,000	$\frac{50}{1}$
“ O.....	Kahl.	1 : 200,000	1 : 4,000	$\frac{50}{1}$
Aurantia (Kryst.).....	G.	1 : 4,000,000	1 : 1,000 (Saturated.)	$\frac{4000}{1} +$
Azur II.....	“	1 : 200,000	1 : 2,000	$\frac{100}{1}$
Chinolinrot.....	Kahl.	1 : 400,000	1 : 40,000	$\frac{10}{1}$
Chrysanilin.....	“	1 : 200,000	1 : 4,000	$\frac{50}{1}$
Coerulein S.....	G.	1 : 100,000	1 : 1,000 (Saturated.)	$\frac{100}{1} +$
Cresylechtviolett.....	“	1 : 400,000	1 : 2,000	$\frac{200}{1}$
Krystallviolett.....	“	1 : 1,000,000	1 : 100,000	$\frac{10}{1}$
“ (Hoechst)....	“	1 : 200,000	1 : 100,000	$\frac{2}{1}$
Dahlia.....	“	1 : 100,000	1 : 10,000	$\frac{10}{1}$
Diamantfuchsin.....	“	1 : 100,000	1 : 4,000	$\frac{25}{1}$
Heliotrop.....	Kalle.	1 : 4,000,000	1 : 8,000	$\frac{500}{1}$
Indaminblau N extra.....	Kahl.	1 : 200,000	1 : 2,000	$\frac{100}{1}$
Janusgrün.....	G.	1 : 400,000	1 : 8,000	$\frac{50}{1}$
Magentarot.....	“	1 : 200,000	1 : 5,000	$\frac{40}{1}$
Mauvien.....	“	1 : 400,000	1 : 1,000 (Saturated.)	$\frac{400}{1} +$
Methylene blue.....	Merck.	1 : 1,000,000	1 : 2,000	$\frac{500}{1}$

\* In the tables, G. indicates Grübler; Kahl., Kahlbaum; Kalle, Kalle and Company; By., Bayer and Company. The other names are self-explanatory.

TABLE I—*Concluded.*

Designation.	Source.	Tubercle bacillus growth full at.	Typhoid bacillus growth full at.	Typhoid bacillus Tubercle bacillus
Methylengrün.....	G.	1 : 200,000	1 : 2,000	$\frac{100}{1}$
Methylenviolett.....	"	1 : 2,000,000	1 : 4,000	$\frac{500}{1}$
Methylviolett (Kryst.).....	Kahl.	1 : 200,000	1 : 100,000	$\frac{2}{1}$
" B.....	G.	1 : 200,000	1 : 20,000	$\frac{10}{1}$
" 5B.....	"	1 : 200,000	1 : 40,000	$\frac{5}{1}$
" 6B extra.....	Kahl.	1 : 400,000	1 : 40,000	$\frac{10}{1}$
Neutral blue W.....	Kalle.	1 : 200,000	1 : 1,000 (Saturated.)	$\frac{200}{1} +$
" red.....	G.	1 : 400,000	1 : 100 (Incomplete.)	$\frac{4000}{1} +$
Nilblau (sulfat).....	"	1 : 2,000,000	1 : 4,000	$\frac{500}{1}$
Orcein.....	"	1 : 100,000	1 : 800	$\frac{125}{1}$
Orcin.....	"	1 : 100,000	1 : 2,000	$\frac{50}{1}$
Phosphin I.....	Unknown.	1 : 400,000	1 : 2,000 (Saturated.)	$\frac{200}{1} +$
" 3R.....	Kahl.	1 : 400,000	1 : 8,000	$\frac{50}{1}$
Resorcin fuchsin.....	G.	1 : 100,000	1 : 2,000	$\frac{50}{1}$
Rosanilin-acetat.....	Kahl.	1 : 200,000	1 : 4,000	$\frac{50}{1}$
Safranin 3B.....	Kalle.	1 : 2,000,000	1 : 4,000	$\frac{500}{1}$
" base.....	Unknown.	1 : 1,000,000	1 : 8,000	$\frac{125}{1}$
" MN.....	Badische.	1 : 2,000,000	1 : 4,000	$\frac{500}{1}$
" rein.....	G.	1 : 400,000	1 : 8,000	$\frac{50}{1}$
Spiller's purple.....	"	1 : 200,000	1 : 200,000	$\frac{1}{1}$



TABLE II.

*Azo-Dyes Strongly Restraining Growth of the Tubercle Bacillus.*

Designation.	Source.	Tubercle bacillus growth full at.	Typhoid bacillus growth full at.	$\frac{\text{Typhoid bacillus}}{\text{Tubercle bacillus}}$
Alkaligrün.....	G.	1 : 200,000	1 : 200 (Saturated.)	$\frac{1000}{1} +$
Anilingelb.....	"	1 : 100,000	1 : 4,000	$\frac{25}{1}$
Benzobraun G.....	By.	1 : 200,000	1 : 200 (Saturated.)	$\frac{1000}{1} +$
Biebricher Scharlach.....	G.	1 : 200,000	1 : 100 "	$\frac{2000}{1} +$
Chromanilbraun R.....	Kahl.	1 : 100,000	1 : 100 "	$\frac{1000}{1} +$
Chrysamin.....	G.	1 : 100,000	1 : 1,000 "	$\frac{100}{1} +$
Chrysoidin.....	"	1 : 100,000	1 : 2,000	$\frac{50}{1}$
" extra.....	Kahl.	1 : 100,000	1 : 2,000	$\frac{50}{1}$
Cloth red 3B extra.....	By.	1 : 200,000	1 : 1,000 (Saturated.)	$\frac{200}{1} +$
" " 3G ".....	"	1 : 200,000	1 : 1,000 "	$\frac{200}{1} +$
Dicyanin.....	Kahl.	1 : 100,000	1 : 1,000 "	$\frac{100}{1} +$
Doppelponceau 4R.....	By.	1 : 100,000	1 : 4,000 "	$\frac{25}{1} +$
Goldorange.....	G.	1 : 200,000	1 : 1,000 "	$\frac{200}{1} +$
Havannabraun I.....	Kahl.	1 : 100,000	1 : 1,000 "	$\frac{100}{1} +$
Helianthin.....	G.	1 : 200,000	1 : 1,000 "	$\frac{200}{1} +$
Indian yellow G.....	By.	1 : 100,000	1 : 1,000 "	$\frac{100}{1} +$
Mandarin G extra.....	Kahl.	1 : 200,000	1 : 200 "	$\frac{1000}{1} +$
Metanilgelb 1a.....	G.	1 : 400,000	1 : 1,000 "	$\frac{400}{1} +$
" extra.....	Kahl.	1 : 400,000	1 : 1,000 "	$\frac{400}{1} +$



TABLE II—*Concluded.*

Designation.	Source.	Tubercle bacillus growth full at.	Typhoid bacillus growth full at.	Tubercle bacillus Typhoid bacillus
Methylorange.....	G.	1 : 200,000	1 : 1,000 (Saturated.)	$\frac{200}{1} +$
Naphtaminbraun 2B.....	Kalle.	1 : 200,000	1 : 200 “	$\frac{1000}{1} +$
“ GX .....	“	1 : 100,000	1 : 1,000 “	$\frac{100}{1} +$
Naphtamin fast black SDE.....	“	1 : 100,000	1 : 1,000 “	$\frac{100}{1} +$
Naphtamingrün A.....	“	1 : 100,000	1 : 1,000 “	$\frac{100}{1} +$
“ AG .....	“	1 : 100,000	1 : 1,000 “	$\frac{100}{1} +$
“ B.....	“	1 : 100,000	1 : 1,000 “	$\frac{100}{1} +$
Naphtaminviolett N.....	“	1 : 100,000	1 : 1,000 “	$\frac{100}{1} +$
Naphthylamine black NR ..	“	1 : 100,000	1 : 200 “	$\frac{500}{1} +$
Orange IV.....	Kahl.	1 : 1,000,000	1 : 1,000 “	$\frac{1000}{1} +$
Pyraminorange RT.....	Badische.	1 : 100,000	1 : 100 (Incomplete.)	$\frac{1000}{1} +$
Säurebraun.....	G.	1 : 100,000	1 : 100 (Saturated.)	$\frac{1000}{1} +$
Tropaeolin OO.....	“	1 : 100,000	1 : 1,000 “	$\frac{100}{1} +$
Wollschwarz.....	“	1 : 400,000	1 : 400	$\frac{1000}{1}$

the methyl violets for example, are no more active than many others whose general action is limited, as represented by the *Bacillus typhosus* results. The power to inhibit the growth of the tubercle bacillus is limited to no general class of coal-tar dye. It is noteworthy that a great many azo-dyes possess this characteristic although few of them are active against *Bacillus typhosus* in a marked degree.

A general view of the results suggests that in the tubercle bacillus we have a bacterium which is highly sensitive to slight modifica-

TABLE III.

*Dyes of Various Groups Restraining Growth of the Tubercle Bacillus Moderately.*

Designation.	Source.	Tubercle bacillus growth full at.	Typhoid bacillus growth full at.	Tubercle bacillus Typhoid bacillus
Anthracenblau.....	G.	1 : 40,000	1 : 1,000 (Saturated.)	$\frac{40}{1} +$
Brillantkresylblau.....	"	1 : 40,000	1 : 1,000	$\frac{40}{1}$
Curcumein N.....	"	1 : 40,000	1 : 1,000 (Saturated.)	$\frac{40}{1} +$
Echtrot.....	"	1 : 20,000	1 : 1,000   "	$\frac{20}{1} +$
Indulin.....	"	1 : 20,000	1 : 1,000   "	$\frac{20}{1} +$
Isaminblau 8B.....	Cassella Color Co.	1 : 20,000	1 : 100   "	$\frac{200}{1} +$
Kresofuchsin.....	G.	1 : 20,000	1 : 1,000   "	$\frac{20}{1} +$
Malachitgrün Ia.....	"	1 : 20,000	1 : 4,000	$\frac{5}{1}$
Muscarin.....	"	1 : 40,000	1 : 1,000 (Saturated.)	$\frac{40}{1} +$
Nachtblau.....	"	1 : 20,000	1 : 1,000   "	$\frac{20}{1} +$
Naphtalinrot.....	"	1 : 20,000	1 : 1,000   "	$\frac{20}{1} +$
Pararosanilin chlorhydrat. .	Kahl.	1 : 40,000	1 : 2,000	$\frac{20}{1}$
Pyronin.....	G.	1 : 20,000	1 : 4,000	$\frac{5}{1}$
Rhodamin B.....	Kahl.	1 : 20,000	1 : 100 (Incomplete.)	$\frac{200}{1} +$
Victoriablau B.....	"	1 : 20,000	1 : 10,000	$\frac{2}{1}$
"       4R.....	G.	1 : 20,000	1 : 10,000	$\frac{2}{1}$

TABLE IV.

*Azo-Dyes Restraining Growth of the Tubercle Bacillus Moderately.*

Designation.	Source.	Tubercle bacillus growth full at.	Typhoid bacillus growth full at.	Tubercle bacillus Typhoid bacillus
Bordeaux BX.....	By.	1 : 20,000	1 : 100 (Saturated.)	$\frac{200}{1} +$
Chrysamin G.....	"	1 : 20,000	1 : 1,000 "	$\frac{20}{1} +$
Diamantschwarz PV.....	"	1 : 20,000	1 : 200 "	$\frac{100}{1} +$
Doppelponceau 2R.....	"	1 : 20,000	1 : 1,000 "	$\frac{20}{1} +$
Naphtaminschwarz 2RE....	Kalle.	1 : 20,000	1 : 100 "	$\frac{200}{1} +$
" 3RE....	"	1 : 20,000	1 : 2,000	$\frac{10}{1}$
Naphtaminblau 12B.....	"	1 : 20,000	1 : 100 (Saturated.)	$\frac{200}{1} +$
" BE.....	"	1 : 20,000	1 : 100 "	$\frac{200}{1} +$
" BXG.....	"	1 : 20,000	1 : 100 "	$\frac{200}{1} +$
" BXR.....	"	1 : 20,000	1 : 100 "	$\frac{200}{1} +$
Naphtaminbraun H.....	"	1 : 20,000	1 : 1,000 "	$\frac{20}{1} +$
" RB.....	"	1 : 40,000	1 : 400 "	$\frac{100}{1} +$
Naphtamindirektschwarz C.	"	1 : 20,000	1 : 1,000 "	$\frac{20}{1} +$
Naphtamindirektschwarz RWK.....	"	1 : 20,000	1 : 400 "	$\frac{50}{1} +$
Naphtaminechtscharlach B..	"	1 : 40,000	1 : 1,000 "	$\frac{40}{1} +$
" R.	"	1 : 40,000	1 : 1,000 "	$\frac{40}{1} +$
Naphtaminorange 2R.....	"	1 : 40,000	1 : 1,000 "	$\frac{40}{1} +$
Naphtaminrot H.....	"	1 : 40,000	1 : 1,000 "	$\frac{40}{1} +$
Naphtylamingelb.....	G.	1 : 40,000	1 : 4,000	$\frac{10}{1}$

TABLE IV—*Concluded.*

Designation.	Source.	Tubercle bacillus growth full at.	Typhoid bacillus growth full at.	Tubercle bacillus Typhoid bacillus
Neucoccin.....	Kahl.	1 : 20,000	1 : 100 (Incomplete.)	$\frac{200}{1} +$
Purpurin sicc. opt.....	G.	1 : 20,000	1 : 1,000 (Saturated.)	$\frac{20}{1} +$
Roseazurin G. ....	By.	1 : 20,000	1 : 4,000 “	$\frac{5}{1} +$
Sulfonazurin D. ....	“	1 : 20,000	1 : 1,000 “	$\frac{20}{1} +$
Sulfocyanin G. ....	“	1 : 40,000	1 : 1,000 “	$\frac{40}{1} +$
Wollschwarz 4B.....	Kahl.	1 : 20,000	1 : 200 “	$\frac{100}{1} +$

tions of growth conditions and that we are dealing with a reaction here which bears no real relation to the more usual forms of disinfectant action. This may be the case, but the explanation is hardly constructive. We hope to have for presentation in the near future a series of tests with the pneumococcus carried out on similar lines. It is apparent from what we have done with the pneumococcus that sensitiveness to reaction and exact constitution of the culture medium does not imply sensitiveness to the inhibiting action of dyes as we have developed it in the case of the tubercle bacillus.

Many of these dyes, in fact by far the larger number, stain the growing membrane with greater or less intensity. The individual bacilli in the membrane are much less frequently stained and then only by concentrations much greater than those required to inhibit growth. The restraint of growth seems in no way related to the staining of the membrane.

The staining of the membrane may in some instances possibly render the figures inaccurate. In the case of dyes exhibiting great activity, such as heliotrope, methylene blue, and others, the membrane absorbs the dye from weak solutions so that the concentration in the immediate vicinity of the bacilli is much higher than the dilution number would imply. Whether this increased concentration

TABLE V.

*Weaker Dyes in Concentrations of 1:1,000 to 1:10,000 Showing Full Growth of the Tubercle Bacillus.\**

Designation.	Source.	Designation.	Source.
Acridinrot.....	G.	Naphtamindirektschwarz	
Alizarin red.....	Eimer and Amend.	EK.....	Kalle.
Alizarincyamin.....	Kahl.	Naphtamindirektschwarz O..	"
Alkaliblau 3B.....	"	Naphtamindirektbraun 2R..	"
Anilinblau.....	G.	" GR..	"
Aniline blue black.....	"	" V....	"
Azosaureblau 2G.....	By.	Naphtamine fast black KS	"
Azoblau.....	G.	Naphtamingrün AB.....	"
Bayerischblau.....	Kahl.	" TE.....	"
Benzoolive.....	By.	Naphtaminorange FB.....	"
Blauschwarz.....	G.	Naphtaminscharlach B.....	"
Bordeaux G.....	By.	Naphtaminviolett BE.....	"
Brilliantcrocein 3BA.....	"	Naphtholgelb S.....	G.
Chromgelb D.....	"	Naphtholgrün B.....	"
Congo-corinth.....	G.	Naphtholorange.....	Kahl.
Congo red.....	Unknown.	Naphtholschwarz B.....	"
Congorubin.....	Kalle.	Naphtylaminschwarz BN...	Kalle.
Echtgrün.....	G.	" BNN.	"
Heliotrop B.....	Kalle.	" BOO.	"
Isaminblau 6B.....	Cassella	" NA..	"
" R.....	Color Co.	Nigrosin.....	G.
Jodgrün.....	G.	Patentblau A.....	Kahl.
Lichtgrün.....	"	Phloxinrot.....	G.
Magdalarot echt.....	"	Ponceau PR.....	"
Methylgrün rein OO.....	"	" (Kryst.) 6 RF .....	Kahl.
Naphtaminschwarz GE.....	Kalle.	Resedagrün.....	"
" H.....	"	Rosazurin B.....	G.
" HRE.....	"	Rose bengale.....	"
" 4RE.....	"	Säuregrün.....	"
Naphtaminblau 2BK.....	"	Säureviolett 6B.....	Kahl.
" 3RE.....	"	" (Kühne).....	G.
Naphtaminbrillantblau BW..	"	Sulfonsäureblau R.....	By.
" G.....	"	Sulforhodamin.....	Metz and Co.
Naphtaminbraun 8B.....	"	Thiazinbraun.....	G.
" 4G extra...	"	Tropaeolin.....	Kahl.
		Trypanrot.....	G.
		Victoriascharlach 4R.....	Kahl.
		Violetttschwarz.....	G.

\* The tubercle bacillus factor is indeterminable, except in a few instances, because of the inactivity of the dyes against the typhoid bacillus.

TABLE VI.

*Very Inactive. Full Growth at 1:1,000 or Stronger.*

Designation.	Source.	Designation.	Source.
Äthylgrün (Kryst.).....	Kahl.	Fast brown 26048.....	By.
Aniline red.....	Merck.	“ yellow extra.....	“
Azocarmin.....	G.	Flavazin G.....	Kahl.
Azoeosin.....	By.	Fluoresceïn.....	G.
Azogrenadin L.....	“	Fuchsin S.....	“
Azolitmin.....	A. H. Thom- as and Co.	Gallein (sicc.).....	“
Azorubin S.....	Kahl.	Gentianablau 6B.....	Kahl.
Baumwollblau.....	“	Isaminblau B.....	Cassella Color Co.
Benzoazurin.....	G.	Methyleosin.....	G.
“ G.....	Kalle.	Naphtaminschwarz RGE....	Kalle.
Benzopurpurin.....	G.	Naphtaminblau 7B.....	“
Benzo sky blue.....	By.	“ 2BL.....	“
Bittermandelölgrün.....	G.	“ JE.....	“
Bleu de Lyon.....	“	Naphtaminbrillantblau B... “ BWO	“
Bordeaux R.....	Kahl.	Naphtamintiefschwarz HW.	“
Brillantazurin 5G.....	By.	Naphtaminechtschwarz SE..	“
Brillantgrün.....	Kahl.	“ TE.....	“
“.....	G.	Naphthylaminschwarz 10B..	By.
Brillantschwarz.....	“	Orange G.....	G.
Carminic acid Ia.....	“	Oxaminviolett.....	Badische.
Chinablau.....	“	Patentsäure rubin.....	G.
Chinagrün.....	“	Ponceau 3R.....	Kahl.
Chinolingelb (wasserlöslich)..	Kahl.	“ 4R.....	“
Chinolingelb.....	Uncertain.	Rapid filter gelb.....	“
“.....	Badische.	“ “ grün I.....	“
Chloraminorange G.....	By.	“ “ rot I.....	“
Chromotrop.....	G.	Rosazurin G.....	G.
“ 2R.....	Kahl.	Rubin S.....	“
Crocein.....	G.	Säurecyanin G.....	Kahl.
Diaminblau 3B.....	Kahl.	Säureschwarz B.....	“
“ B.....	“	Säureviolett 1897.....	G.
Direktblauschwarz 2B.....	By.	Tartrazin.....	By.
Direktgelb R.....	“	Thiazinrot.....	G.
Echtröt D.....	Kahl.	Thiazolgelb.....	Kahl.
Eosin (bläulich).....	“	Victoria violet 4BS extra....	By.
“ (clairl.).....	G.	Wasserblau.....	G.
“ (gelblich).....	Kahl.	“ 3B.....	Kahl.
“ (rein franz.).....	G.	“ 6B.....	“
Erythrosin.....	“		



is effectively applied against the growth of the microorganism cannot be determined.

The figures presented above, studied in conjunction with what is known of the chemical constitution of the dyes, are suggestive. The quality of the material does not permit accurate judgment and as we expect to have ready shortly similar observations made with especially prepared or purified substances much more suited for these considerations, this discussion will be deferred for the present.

An important question raised by these observations is the relation between the capacity to restrain growth as shown above, and true disinfectant action or killing power of the substances in question. It has been repeatedly shown that in the case of true disinfectants their capacity to restrain growth is roughly proportional to their killing power. In the tables it appears that the dyes which have long been recognized to have disinfectant action, certain of the triphenyl methanes particularly, belong to the group of substances with a high power to restrain the growth of the tubercle bacillus. In all previous consideration of this question, the starting point has been the substance whose lethal action was known. That there might be substances having marked capacity to restrain growth with no corresponding capacity to kill does not seem to have occurred to previous investigators in this field.<sup>12</sup> Contrasting the great inhibitory power of methylene blue in our hands and the low killing power of this dye in the work of DeWitt with the previous observations on this relation, we may safely conclude that there is no close or constant correlation between the two forms of action. Special work directed towards this point is required before a precise conclusion can be reached.

#### SUMMARY.

The power of a large number of aniline dyes to restrain the growth of *Bacillus tuberculosis* and *Bacillus typhosus* has been determined. Many substances have been found with especial restraining power

<sup>12</sup> Gotschlich, E., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, iii, 479, states that formaldehyde has, as contrasted with other disinfectants of similar lethal value, a high capacity to inhibit growth.

for the tubercle bacillus under the conditions of the test. This capacity to restrain growth in the case of the tubercle bacillus apparently bears no simple relation to true disinfectant action. Opinion as to whether the active substances exert a truly specific activity against the tubercle bacillus or whether the activity is determined by the peculiar conditions imposed by the growth of this bacterium as a surface membrane are left for future consideration.



## PROTEOSE INTOXICATIONS AND INJURY OF BODY PROTEIN.

### I. THE METABOLISM OF FASTING DOGS FOLLOWING PROTEOSE INJECTIONS.

BY G. H. WHIPPLE, M.D., AND J. V. COOKE, M.D.

*(From the George Williams Hooper Foundation for Medical Research, and the Department of Pathology of the University of California Medical School, San Francisco.)*

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In recently reported experiments (1), we have pointed out that the non-protein nitrogen of the blood may show a considerable increase above normal due to intestinal obstruction or the presence of a closed intestinal loop. A definite rise in blood non-protein nitrogen may be found in association with general peritonitis or septicemia, and an acute rise can be demonstrated following an injection of a toxic proteose (1). We have repeatedly observed an increase of over 100 per cent non-protein nitrogen in the blood within 3 or 4 hours following an intravenous injection of 100 to 300 mg. of purified proteose obtained from closed intestinal loops. Such an increase can scarcely be accounted for by any kidney retention of non-protein nitrogen. This observation suggested strongly that there might be a marked protein or tissue disintegration associated with proteose intoxications.

The experiments reported below give a suitable explanation for the high non-protein blood nitrogen in the acute intoxications. They show that the injection of a toxic proteose will cause a profound upset in the body metabolism. Fasting dogs which have reached a base-line of nitrogen elimination when given a single proteose injection will show a remarkable increase in total urinary nitrogen—often an increase of 4 to 6 gm. of nitrogen above the base-line level. This indicates a considerable destruction of body protein,

which is effected by the poison. With a distinct proteose intoxication, there are evident clinical signs and symptoms, vomiting, diarrhea, low blood pressure, and a temperature reaction. Curiously enough the greatest amount of nitrogen is usually excreted during the second 24 hours following the injection when the dog may appear in approximately a normal condition. Evidently the tissue disintegration produced by the proteose is not so prompt as the clinical reaction or there is considerable delay in elimination of the end-products which result from this tissue injury. The reaction often lasts 4 to 6 days after a single injection, and indicates a profound disturbance of protein metabolism.

### *Methods.*

All the experiments were performed upon fasting dogs. The tables show the urinary and fecal nitrogen during fasting periods. The dogs were kept in standard metabolism cages with wire mesh floors and metal bottoms having a sharp pitch toward the collecting spout. This type of cage assured the minimum amount of urine evaporation. The urine flowed into a glass container in which were placed 2 or 3 cc. of glacial acetic acid to neutralize any chance alkaline reaction of the urine. At 10 a.m. each day the dogs were catheterized, and the bladder was rinsed out and left empty. The cages were then cleaned, and the cage washings carefully saved to be used in making up the urine to a unit volume of 1 or 2 liters. If solid feces were passed, the material was kept separate from the cage washings, but semifluid or fluid feces were combined with the cage washings and analyzed as a unit. The total nitrogen of urine and feces was determined in the usual way by the Kjeldahl method. All determinations were done in duplicate.

It will be noticed that the dogs were not given uniform amounts of water each day, as is desirable to obtain a uniform basal nitrogen elimination during periods of fasting. There were two reasons for this. Dogs with proteose intoxications of various kinds are apt to vomit, and will vomit very promptly any fluid given by stomach, which tends to complicate the picture. Secondly, it is not necessary to have these dogs on a uniform base-line of nitrogen elimination, because the reactions following the proteose injections are so marked. The dogs were permitted to drink water as they desired, but no food of any kind was given during the experimental period, unless a note is made to that effect.

Proteose injections were usually given about noon or shortly after that time. In all the experiments the dogs were lightly anesthetized for about 5 minutes to facilitate the injection of the proteose solution into the jugular vein. Injections were made rather slowly by means of a needle in the vein, but were usually completed during an interval of 3 minutes.

The proteose was obtained from human or dog material, as indicated in the various tables. The preparation and isolation of such proteose material have been described in another publication (2). The fresh material from a case of obstruction or a closed intestinal loop may in some cases be diluted with distilled water, if it is too thick to handle easily, and then precipitated with five volumes of 95 per cent alcohol. This precipitation is complete in a few hours, and the precipitate can be used then or after a period of weeks, as no change occurs in this alcoholic mixture. The precipitate is collected by centrifugalization and dissolved in distilled water usually to a volume three times greater than the volume of fluid originally precipitated by the alcohol. This solution may be rich in albumins. It is brought to a boil, and a few drops of acetic acid are added to promote coagulation, a reaction faintly acid to litmus. The coagulum is removed by filtration, leaving a clear, slightly opalescent fluid. This is made faintly alkaline to litmus, brought to a boil, and cooled. This may give a faint precipitate, which is removed by filtration. The solution contains a toxic proteose, and may be given as such intravenously. If desired, this solution may be again precipitated by alcohol, collected in the centrifuge, dissolved in water, boiled in a faintly acid solution, filtered, and made up to faint alkalinity. Or further purification can be accomplished by one-half saturation with magnesium sulfate, removal of the precipitate, solution in water, and dialysis to remove the excess of salt. The toxic fraction in this material is easily precipitated by alcohol or magnesium sulfate, and the chemical reactions as well as its biological characteristics indicate that this substance is a primary proteose (2).

#### EXPERIMENTAL OBSERVATIONS.

*Dog 16-110.*—(Table I.) Sturdy, black cocker spaniel; adult male. February 11, 1916. Isolation in metabolism cage with no food. The dog was normal, and no injections of proteose had been given previously.

The proteose was obtained from a human case of intestinal obstruction at operation. This fresh material was precipitated with alcohol, and the proteose isolated in the usual manner. The same material was used in both injections (Table I), in the first injection 25 cc., and in the second injection 35 cc. of a standard solution. The clinical reaction is much the same in the two experiments, showing some increased tolerance for the poison. The increase in nitrogen elimination lasts longer following the first injection, but the total increase above the mean is about the same in both instances, about 6 gm. of nitrogen.

Dog 16-109 (Table II) gives a better illustration of the tolerance to



proteose injection which may develop promptly following the initial injection and protect the body against the injury which is evidenced by clinical symptoms and the rise in the curve of urinary nitrogen.

Dog 16-110 also shows a distinct diuresis following the proteose injection. The volume output of urine was not followed carefully in this experiment, but in a later experiment we find that this dog put

TABLE I.  
*Proteose Injections. Total Nitrogen Elimination.*  
*Dog 16-110. Normal.*

Day.	Urine.		Feces nitrogen.	Weight.	Remarks.
	Nitrogen.	Amount.			
	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>lbs.</i>	
1	2.91	—	0	29.0	Fasting previous 4 days.
2	3.02	—	0.62*	28.5	
2	Proteose from human intestinal obstruction. Moderate intoxication.				
3	2.06	—	1.09*	27.5	Feces and vomitus. Temperature 38.5°C.
4	5.03	—	0	27.3	Temperature 38.4°C.
5	4.42	800	0	26.8	Diuresis. Dog normal.
6	4.64	550	0	26.5	
7	3.60	550	0	26.1	
8	2.97	—	0	26.1	Temperature 38.3°C.
8	Proteose from human intestinal obstruction. Larger dose and same reaction.				
9	3.64*	—	—	25.5	Definite intoxication.
10	5.82	1,100	0	24.9	Diuresis. Temperature 38.3°C.
11	5.11	700	0	24.5	Clinical improvement.
12	3.51	—	0	24.1	Dog normal.
13	2.66	—	0.58	24.0	
14	2.82	—	0	23.8	Normal urine output (200 cc. average).

\* Feces and cage washings.

out about 200 cc. of urine per 24 hours. This gives sufficient control to show the extreme diuresis during the period of high nitrogen output following the proteose injections.

*Dog 16-109.*—(Table II.) Strong, old fox-terrier; male. This dog had received no proteose injection prior to the experiments, and had

been normal during a considerable period of observation in the laboratory. The details of the proteose injections are given in Table II. The proteose used in both injections was obtained from a closed loop of dog's intestine, precipitated by alcohol, and prepared in the usual way. The first injection of 25 cc. of a standard solution caused a moderate clinical intoxication with considerable increase in the total nitrogen elimination as usual. The usual temperature reaction

TABLE II.  
*Proteose Injections. Total Nitrogen Elimination.*  
*Dog 16-109. Normal.*

Day.	Urine.		Feces nitrogen.	Weight.	Remarks.
	Nitrogen.	Amount.			
	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>lbs.</i>	
1	3.76	—	0	22.3	Fasting previous 4 days. Temperature 38.9°C.
2	3.08	—	0.53	22.0	
2	Proteose from dog intestinal loop. Moderate intoxication.				
3	3.76	—	0.22*	21.5	Temperature 38.7°C.
4	4.53	—	0	21.0	
5	3.58	275	0	20.8	
6	3.33	340	0	20.4	
7	3.40	250	0	20.0	
8	3.09	—	0.39	19.8	
8	Proteose from dog intestinal loop. Slight intoxication.				
9	2.56	—	0.85*	19.0	Complete recovery. Temperature 38.7°C.
10	3.52	600	0	18.8	
11	2.91	300	0	18.6	
12	2.94	—	0	18.3	

\* Feces and cage washings including vomitus.

(Table VI) occurred, and there was repeated vomiting and slight diarrhea. The second injection caused only slight clinical intoxication, yet 40 cc. of the same standard solution were injected intravenously. There was a slight temperature reaction and only a trivial increase in urinary nitrogen. This is a good example of the tolerance which may develop following proteose injections. It should be emphasized that there is nothing specific about this immunity or tolerance to proteose injection.

*Dog 16-46.*—(Table III.) Lean, active, mongrel fox-terrier; adult male.

November, 1915. The dog was injected with a proteose obtained from the pancreas, and was slightly intoxicated. During this time and for several weeks later the animal was suffering from a moderately severe attack of distemper. A good recovery from the distemper was made in 2 or 3 weeks.

February 27, 1916. Dog in good condition. The details of the experiment are given in Table III. The first injection of proteose from a human case of general peritonitis gave no clinical symptoms of intoxication, but we cannot state whether there might not have been a rise in urinary nitrogen on the 2nd day following, as, unfortunately, a second dose of proteose from a closed intestinal loop was given. This injection caused a slight amount of clinical intoxication but a considerable rise of urinary nitrogen, which persisted for a long time.

TABLE III.

*Proteose Injections. Total Nitrogen Elimination.**Dog 16-46. Immune.*

Day.	Urine.		Feces nitrogen.	Weight.	Remarks.
	Nitrogen.	Amount.			
	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>lbs.</i>	
1	2.83	—	0	25.4	Fasting previous 5 days.
2	2.95	—	0.98*	25.0	
3	2.38	—	0	24.5	Temperature 38.2°C.
3	Proteose from human peritonitis. No intoxication.				
4	2.41	—	0.67*	24.3	Temperature 38.3°C.
4	Proteose from dog intestinal loop. Slight intoxication.				
5	4.05	—	1.13	23.4	Dog active. Temperature 37.8°C. Clinically normal.
6	4.97	—	0.84*	23.1	
7	4.17	—	0	22.5	
8	3.55	—	0	22.0	
9	3.37	—	0	21.9	
10	3.40	—	0	21.8	

\* Feces and cage washings.

In some experiments following a proteose injection, we have found that the curve of urinary nitrogen did not return to the original base-line during the period of observation, in spite of clinical evidence for a complete return to normal. Some of these dogs have been killed, and autopsy showed a small focus in a lung, perhaps due to an old distemper infection. Again an endocarditis has been found in several cases. Some cases may be negative, and we may be forced to assume that the body cells have been so injured that they are unable to repair the injury and return to normal during the period of observation.

TABLE IV.

*Proteose Injections. Total Nitrogen Elimination.*

*Dog 16-114. Slight Distemper.*

Day.	Urine.		Feces nitrogen.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.	gm.	lbs.	
1	4.53	—	0	34.4	Fasting previous 5 days.
2	4.56	180	0	34.0	Distemper suspected.
2	Proteose from dog intestinal loop. Moderate intoxication. Vomiting, no diarrhea.				
3	5.71*	180	0	33.8	Temperature 38.6°C. Slight intoxication.
4	5.15	410	0	32.8	Temperature 38.3°C. Diuresis.
5	6.36	—	0	32.1	Dog seems normal.
6	5.14	200	0	31.8	
7	4.91	180	0	31.3	
8	4.71	200	0	31.0	
9	4.50	200	0	30.6	Note the normal after-period (nitrogen = 2.85).

\* Vomitus included.

*Dog 16-114.*—(Table IV.) Large, short-haired, mongrel; adult male. This dog was suspected of having a mild infection of distemper (*Bacillus bronchisepticus*), but the experiment was carried through and no definite clinical symptoms of distemper developed. It was not until a later metabolism period (6 months later) that it was evident that our suspicions were correct. During this last period the

base-line of nitrogen elimination was 2.85 gm. per 24 hours. We have observed several similar cases, and in certain experiments have observed a sharp rise in basal nitrogen elimination with no clinical symptoms, followed after several days of high nitrogen elimination by the usual clinical picture of distemper. This point must be always kept in mind in work of this kind on dogs that are susceptible to distemper.

It should be noted that even with the high basal excretion of urinary nitrogen, the proteose injection causes a rise in the nitrogen curve. This dog shows that the presence of one intoxication causing a high level of nitrogen excretion does not in any way interfere with the reaction following a single proteose injection.<sup>1</sup>

Tables V and VI give the results of three simultaneous experiments with proteose injection. The proteose was obtained in the usual way by alcoholic precipitation of dog intestinal loop fluid, and was further purified by a second alcoholic precipitation. The solution was given intravenously, 1 cc. per pound of body weight, and the clinical reactions can be followed in Table VI. The three dogs, active, strong, mongrel fox-terriers, were all in normal condition. Dogs 16-109 and 16-46 had been injected three times previously with proteose solutions, and were supposed on the basis of other experiments to have a certain degree of tolerance or immunity to subsequent proteose injections. This comes out in a study of these two tables.

The temperature reactions in practically all our experiments were followed as in these experiments (Table VI), but it is not necessary to record these observations, because the reaction following a proteose injection is so constant. These three dogs are given as characteristic examples.

Tables V and VI show several interesting points. They confirm the previous experiments, and show the great rise in total urinary nitrogen which follows an intoxication produced by a proteose. This rise in urine nitrogen may last over several days, whereas the clinical intoxication is obvious for only a day or two, and the febrile reaction is over within 12 hours.

<sup>1</sup> Compare Table I in the following paper, where a chronic intoxication due to an intestinal loop was combined with an injection of proteose.



It is seen that the non-immune dog shows the most intense clinical reaction to the proteose poison, and the highest rise in urinary nitrogen above the mean base-line average. The non-immune dog shows the initial delay in nitrogen elimination, the greatest rise above normal occurring in the second 24 hours. Of the two immune dogs, the larger dog (No. 16-46) shows less clinical intoxication, a higher temperature

TABLE V.

*Proteose Injections. Normal and Immune Dogs. Total Nitrogen Elimination.*

Day.	Dog 16-4. Non-Immune.			Dog 16-109. Immune.			Dog 16-46. Immune.			Remarks.
	Urine nitro- gen.	Feces nitro- gen.	Weight.	Urine nitro- gen.	Feces nitro- gen.	Weight.	Urine nitro- gen.	Feces nitro- gen.	Weight.	
	gm.	gm.	lbs.	gm.	gm.	lbs.	gm.	gm.	lbs.	
1	2.52	0	19.6	2.49	0	19.8	2.30	0	24.5	Fasting previ- ous 4 days.
2	2.46	0	19.5	2.52	0	19.5	2.32	0	24.0	
3	2.52	0	19.4	2.46	0	19.1	2.24	0.36	23.6	
4	2.49	0	19.0	2.62*	±	18.9	2.27	0	23.3	
4	Proteose injection.			Loop fluid reprecipitated by alcohol.			Unit amount per pound			
	of body weight.									
5	3.61	1.09*	18.0	2.49*	±	18.3	4.62*	±	22.5	Vomitus in cage.
6	4.42	0	17.5	3.57	0	17.9	3.22	0	22.0	
7	3.64	0	17.3	3.08	0	17.6	2.66	0	21.9	
8	2.70	0	17.0	2.44	0	17.5	2.58	0	21.6	
9	2.93	0	17.0	2.53	0	17.3	3.01	0	21.6	
Severe intoxication.				Moderate intoxica- tion.			Moderate intoxica- tion.			
Nitrogen excretion above the mean = 5.89 gm.				Nitrogen excretion above the mean = 1.51 gm.			Nitrogen excretion above the mean = 4.69 gm.			

\* Feces and cage washings included.

reaction, and a more prompt elimination of nitrogen. It should be kept in mind that the proteose injection was proportional to body weight, and we must reduce the nitrogen figures of Dog 16-46 by 20 per cent before comparing them with the figures of Dog 16-109 or Dog 16-4. It has been shown in previous communications that dogs show



individual variations in their reaction to proteose injection, and we cannot expect the results to be uniform. It is safe to say that in general the dog which has had previous proteose injections will be less disturbed by a standard unit proteose injection, and will show less increase in urinary nitrogen above the mean base-line excretion

TABLE VI.

*Proteose Injections. Normal and Immune Dogs. Temperature and Clinical Reactions.*

Hour.	Dog 16-4. Non-immune.		Dog 16-109. Immune.		Dog 16-46. Immune.	
	Temperature.	Clinical reaction.	Temperature.	Clinical reaction.	Temperature.	Clinical reaction.
<i>p. m.</i>	°C.		°C.		°C.	
12.30*	37.9		38.4	Feces and vomitus.	38.0	Feces.
1.00	38.3	Vomitus, diarrhea.	38.5	Vomiting.	38.2	Vomiting.
1.30	37.8	More diarrhea.	38.6	Bile-stained vomitus.	39.0	Diarrhea.
2.00	37.9	Diarrhea and tenesmus.	39.1	Pulse pressure fair.	39.4	"
2.30	38.5	Vomiting continues.	39.5		39.8	Pulse pressure good.
3.00	38.9	Gelatinous diarrhea.	39.9	Diarrhea and tenesmus.	40.3	Vomiting.
3.20	39.2	Pulse tension poor.	40.1		40.4	No prostration.
4.00	39.4	Prostration, vomiting.	39.8	Vomiting.	40.6	Slight intoxication.
4.30	39.5	Severe intoxication.	39.6	No prostration.	40.2	
5.00	39.3		39.3	Recovery beginning.	39.7	
6.00	39.4	Clinical improvement.	39.1		38.9	Recovery almost complete.

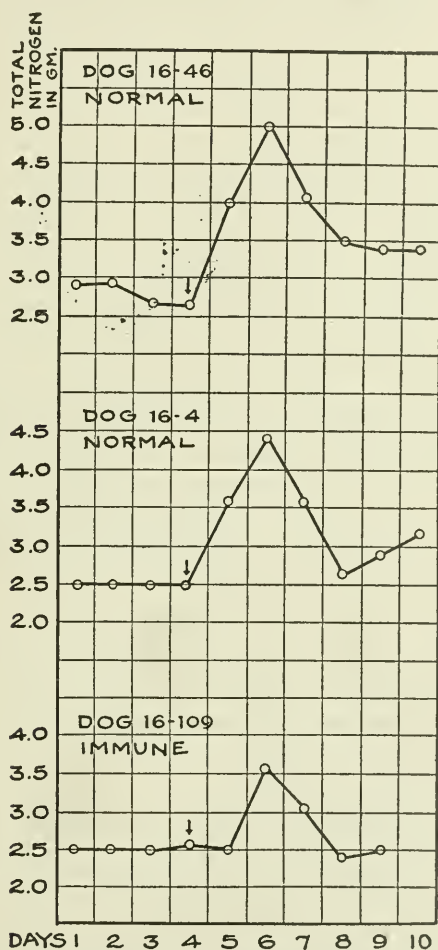
\* A unit amount of pure proteose per pound of body weight was given intravenously at 12.10 to 12.20 p.m.

than the normal control (see also Tables I and II). This comes out in a more striking manner in later experiments. In Dog 16-110,<sup>2</sup> a lethal dose of proteose is survived with a small nitrogen increase,

<sup>2</sup> See Table I of the following paper.

because of the presence in this dog of a closed intestinal loop causing chronic intoxication and a high tolerance to proteose.

The temperature reaction following a proteose injection is the re-



TEXT-FIG. 1. Proteose injections. Total nitrogen elimination. The arrows indicate the injections of proteose.

sultant of two or more factors. There is a tendency for a small dose to cause a certain temperature rise and a larger dose a higher temperature, but a severe intoxication often shows a period of sub-

normal temperature during the time of prostration followed by a rise with clinical improvement (Dog 16-4). Fatal poisoning usually shows a steady fall in temperature to a low level before death. There may be a slight initial rise above normal in these fatal cases.

#### DISCUSSION.

The experiments tabulated above are a unit as regards several points. The fasting dog with a base-line of nitrogen excretion reacts promptly and characteristically to a toxic proteose given intravenously. The clinical reaction is violent and abrupt, but passes off rapidly. The urine shows a great increase above the base-line nitrogen elimination. The rise above normal may be present in mild intoxications on the 1st day or absent at first in severe intoxications. The second 24 hour period usually shows the most marked rise above normal, often twice normal and even three times the normal amount of nitrogen in the urine. The curve of nitrogen excretion does not fall promptly to normal, but declines slowly, and may persist for 4 to 7 days or even longer following a single injection of proteose. This fact indicates a definite tissue or protoplasmic injury which is not adjusted promptly.

There is usually more or less diuresis at this time, and in some experiments a remarkable rise in urine volume (Table I) is noted. The urine volume was not carefully studied in these particular experiments, but this diuresis comes out more clearly in experiments to be published in the near future.

To permit of a clean-cut discussion of this subject of proteose intoxication, it is necessary to recall a few facts which are easy to demonstrate by the injection of a toxic proteose in a normal dog. A small injection of proteose may be given through a hypodermic needle in the jugular vein without anesthesia. The initial reaction is almost instantaneous and within a second or two, even before the injection is completed, the dog will show salivation. Within 5 or 10 minutes the dog may begin to vomit bile-stained mucus. This will continue, depending upon the amount of proteose injected, and feces will soon be passed. Diarrhea may become evident within an hour or less.

The blood pressure reaction may not appear for over an hour, whereupon a progressive fall in blood pressure and temperature may be noted in severe cases. Mucus and blood-tinged diarrhea may appear at this time, especially in cases which end fatally within 6 hours. In some instances the blood pressure reaction may be as prompt as the vomiting. It is possible that the ether anesthesia used in some experiments may explain some of this delay in the toxic reaction.

The blood often becomes incoagulable when tested *in vitro* following a large proteose injection. An excess of antithrombin can be demonstrated in these cases. This is not an absolutely constant finding, and is usually not demonstrable after small doses of proteose, particularly if the injection is made slowly. Too much emphasis should not be placed upon this factor.

A characteristic feature of autopsies of fatal cases of acute proteose intoxication is the splanchnic engorgement (3). This is well shown in the spleen, liver, and pancreas. The mucosa of the small intestine, particularly the duodenum, shows this reaction best—a velvety purple engorged mucosa.

Blood concentration is the rule in almost all cases of acute proteose intoxication. The blood is almost syrupy, and yields only one-fourth or less of its total volume as plasma when drawn into oxalate. Normal dog blood drawn into oxalate (1 part to 9) yields slightly more than one-half by volume of plasma, using the usual speed centrifuge. One's first thought is to explain this blood concentration by the vomiting, diarrhea, and consequent loss of fluid. But it is possible to produce such an acute proteose intoxication that there will be scarcely any loss of fluid by vomiting or diarrhea. A large dose (two or three times a minimum lethal dose) of proteose may be given intravenously and cause death within 2 hours with practically no vomiting and not a trace of feces. These cases may show dry, serous surfaces and a blood which is syrupy and will yield per 100 cc. scarcely 5 to 10 cc. of plasma on centrifuging the oxalated blood. This is almost conclusive proof that much of the circulating fluid has been taken up by the body tissues. This evidence for a definite tissue injury should not be lost sight of in the discussion which follows.

We may now ask: How does the proteose act upon the individual?

Does this toxic proteose act on the blood plasma, on the blood cells, or upon the body tissues, or both plasma and cell protein? That the proteose can cause marked changes in metabolism and all grades of intoxication is obvious.

The work of Jobling, Petersen, and Eggstein (4, 5) and others is usually interpreted to mean that the most important changes take place in the blood plasma. We believe that their work can be interpreted in the light of a profound cell injury and liberation of the ferments into the blood. Experiments with blood serum or plasma *in vitro* do not disprove this point. Their work shows a mobilization of ferments demonstrable in the blood following proteose injections and other intoxications. There is a fluctuation of the ferment-antiferment balance with periods of protease activity, which is believed to act upon serum proteins with the formation of toxic substances or split products.

We wish to point out again the evidence that there is a definite tissue reaction following a large toxic dose of proteose. The blood plasma is greatly concentrated without any marked loss of fluid from the body, and it may be assumed that the body protoplasm has taken up much fluid from the blood. It may be argued that the blood plasma reaction is primary, and the tissue reaction is secondary, or *vice versa*. More evidence is needed to settle this point, and it is possible, if not probable, that both body cells and body fluids react to the initial proteose injection.

The long continued excretion of excess nitrogen after a single proteose injection, a period of 3 to 7 days or even longer, speaks for an essential injury to cell protoplasm somewhere in the body. We have no histological evidence of this injury, but it is now well recognized that a cell may be profoundly disturbed as to function without showing any structural variation from normal which we can recognize with present methods. We have no evidence that any particular organ or tissue is picked out by this toxic proteose, but body cells which are especially rich in ferments may be the most susceptible.

For the sake of visualizing the reaction of this proteose upon cell protoplasm, we may assume that the protoplasm is in delicate equilibrium, perhaps a ferment-antiferment equilibrium or colloidal dis-



persion equilibrium. The toxic proteose may shock or disturb this delicate physical balance, and a multitude of reactions may take place. The protoplasm may take up available water—hydration of a colloid. This phenomenon may be the cause or effect of the protoplasmic injury, but it is fairly definite. There is no evidence that this reaction is reversible, but the chain of events when once started follows a well recognized sequence. It has been shown (6) that a short time after a proteose injection the blood of the poisoned dog is inert toward a normal dog. This again emphasizes the body cells as the elements attacked by the proteose, which is so rapidly withdrawn from the blood stream.

Whatever the word picture we use—ferment-antiferment balance, physical or colloid balance, or any other—we mean that there is evidence of a direct injury of cell protoplasm by this toxic proteose. No efficient method of restoring this balance or undoing the cell injury has been discovered. We know that the body is able to bring conditions back to normal if the shock has not been too great, but we do not understand this mechanism. We have previously submitted evidence (7) again in favor of the activity of the tissue and organ cells. Repeated injections of sublethal doses of proteose bring about a certain degree of tolerance or immunity in dogs (7). The body fluids of these dogs are inactive toward toxic proteoses, but the organ emulsions and ferment-containing organ extracts of these dogs destroy *in vitro* the toxic proteoses. It is extremely important to study every phase of this reaction in the immune animal, for we may by such means gain an understanding of the mechanism of body defense. This knowledge may be of great value in the treatment of acute intoxications.

It has been noted that following an injection of proteose, there may be a latent period of an hour or more before the grave toxic reaction with prostration sets in. The blood removed at this time from an intoxicated dog is inert toward a normal animal. These two facts suggest an initial injury to cell protoplasm, which starts some intracellular reaction resulting in protein splitting with the formation of more toxic substances and the final overwhelming of the host. This may be spoken of as a vicious circle, where the equilibrium between the arc of the vicious circle and the straight line of normal



balance is extremely delicate. If the body reserve cannot restore this balance, the cells may actually form substances from their own protoplasm which are fatally toxic. The physical condition of the cell may be so changed from normal without any histological change that cell life and metabolism are no longer possible.

It is well known that a proteose injection will cause great destruction of white cells, but no appreciable red cell injury. It may be assumed that this is primary or secondary to the other reactions which have been mentioned. It is also possible to look upon this as further evidence of cell injury by this toxic proteose—an upset in the delicate protein equilibrium in these cells with disintegration and perhaps further production of toxic split products. It is known that white cells are rich in ferments,—so too are liver cells and the epithelial cells of the pancreas and intestinal mucosa.

The tabulated experiments of this paper add some facts concerning the tolerance or immunity which develops in dogs after repeated sublethal doses of proteose. It is clear that the second or third unit dose of proteose will cause, as a rule, much less violent clinical reactions (Tables II and VI). In general, we may say that the immune dog will put out less urinary nitrogen than a similar control dog after a unit injection of toxic proteose. The clinical features and excess of nitrogen elimination are usually in accord;<sup>2</sup> the reactions tend to run parallel, but we are inclined to believe that the curve of nitrogen excretion gives a more accurate index of body injury caused by the proteose than do the clinical reactions.

#### SUMMARY.

Proteose injections in dogs cause vomiting, diarrhea, temperature reactions, low blood pressure, prostration, and, after large doses, an excess of antithrombin with incoagulable blood.

A single proteose injection, for example one-half a lethal dose, causes abrupt clinical reactions in a normal dog with apparent complete recovery within 24 to 48 hours.

The nitrogen elimination curve in a fasting dog under such conditions shows a great rise in total urinary nitrogen. The apex of the curve usually falls during the second 24 hour period following

the injection. This rise may be over 100 per cent increase above the mean base-line nitrogen level. It does not fall promptly to normal but declines slowly in 3 to 5 days or more toward the original base-line (Text-fig. 1). This speaks for a definite cell injury with destruction of considerable protein substance due to a single proteose injection. The disturbance of cell equilibrium is not rapidly or promptly restored to normal.

A dog which has received previous proteose injections is partially immune or tolerant to subsequent injections of proteose. These dogs, as a rule, show less intense clinical reactions and less rise in the curve of nitrogen elimination following a unit dose of standard proteose as compared with normal or non-immune controls.

The proteose used in these experiments was prepared as described from material obtained in cases of intestinal obstruction or of closed intestinal loops.

These experiments explain the sharp rise in blood non-protein nitrogen which follows within a few hours the injection of a toxic proteose. They also point to the correct explanation of the high non-protein nitrogen of the blood found in intestinal obstruction or with closed intestinal loops.

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# PROTEOSE INTOXICATIONS AND INJURY OF BODY PROTEIN.

## II. THE METABOLISM OF DOGS WITH DUODENAL OBSTRUCTION AND ISOLATED LOOPS OF INTESTINE.

BY G. H. WHIPPLE, M.D., J. V. COOKE, M.D., AND T. STEARNS, M.D.

*(From the George Williams Hooper Foundation for Medical Research and the Departments of Pathology and Surgery of the University of California Medical School, San Francisco.)*

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The results which follow the injection of toxic proteoses in normal and immune dogs have been reported in a previous communication.<sup>1</sup> The proteose material was obtained from intestinal obstruction or from closed loops of the intestine. The present paper deals with the reaction which develops in the dog following intestinal obstruction of a certain type or the isolation of closed intestinal loops. It is clear that the curves of nitrogen elimination show the same high rise above normal following intestinal obstruction as recorded above, as after an injection of a toxic proteose. We have long believed that the intoxication associated with isolated intestinal loops and intestinal obstruction is due to the absorption from the intestinal mucosa of a toxic proteose similar to the proteose which can be isolated from the closed loops or obstructed intestine. These experiments give further support to this belief, and add other facts which are of value for a proper understanding of the various proteose intoxications.

### *Methods.*

Dogs were used in all the experiments. All operations were done under complete surgical ether anesthesia with the usual aseptic technique. The various procedures for the collection of urine, analysis, etc., are described in detail in the preceding paper.

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<sup>1</sup> Whipple, G. H., and Cooke, J. V., *J. Exp. Med.*, 1917, xxv, 461.

## EXPERIMENTAL OBSERVATIONS.

*Dog 16-110.*—(Table I.) Strong, black cocker spaniel; adult male.<sup>2</sup>

Feb. 15, and Feb. 21, 1916. Proteose injections were given as described. Both caused a definite intoxication, but the second dose was larger without causing any more marked intoxication, an evidence of established tolerance.

Mar. 29. The dog is completely recovered from previous experiments, and should have a moderate tolerance or immunity against proteose. It has been established that these dogs survive a closed loop for a longer time than a non-immune animal (1). The metabolism experiment was started and carried on as usual (Table I).

Mar. 31. Ether anesthesia and operation to produce the usual closed loop of jejunum. The larger part of the jejunum was isolated, the ends turned in, and the small intestine united around it by an end to end anastomosis. Time of operation,  $1\frac{1}{2}$  hours. The dog made a good recovery, and gave no signs of acute intoxication as did Dog 16-156 (Table II). There was no complicating vomiting and little feces.

Apr. 16. Dog shows no clinical signs of intoxication. Visible peristalsis seen for the first time. This indicates some filling of the closed loop.

Apr. 20. Several feedings (Table I) of sugar and vegetable fat were given to study the amount of protein-sparing effected by this means. These experiments and others indicate that under this condition of chronic intoxication from a closed loop, the protein-sparing action of carbohydrates is less marked than in a normal dog.

May 1. Condition unchanged. Visible peristalsis is not conspicuous, but it is easy to see. Intravenous injection of a standard proteose obtained from a closed loop of intestine.

A control dog was given the same dose per pound of body weight with a rapidly fatal result. The autopsy findings were typical of acute proteose intoxication.

This dog (No. 16-110) had the most marked tolerance to proteose which we have noted in all our experiments. He also survived a closed loop of the jejunum longer than any animal in a large series, so we may assume that he was highly tolerant because of the continued slow intoxication from the closed intestinal loop. He was protected against the acute initial intoxication of the loop by the previous injections of proteose.

This lethal dose of proteose was given to Dog 16-110 without any definite signs of intoxication. Following the injection there was

<sup>2</sup> The first proteose injections in this dog are recorded in Table I of the preceding paper.

a slight febrile reaction and one attack of vomiting. The dog passed soft feces, but there was no diarrhea. The pulse pressure was excellent. The elimination of nitrogen in the urine indicates the same lack of definite reaction, and we note only a trivial rise in total nitrogen. Compare with this the reaction of this dog when injected with a fraction of a lethal dose of proteose obtained from a case of human intestinal obstruction.<sup>2</sup>

May 6. Ether anesthesia, operation, and removal of the closed loop of jejunum. The dog recovered and is in normal condition at present. The closed loop contained thick, pasty material in considerable amount. This material was diluted, and preserved for further study. Total nitrogen of loop fluid, 5.21 gm. This should be compared with the fluid which accumulated rapidly in a similar loop in Dog 16-156 (Table II). This fluid was more abundant as the loop was longer and more acutely distended (Dog 16-156), yet it contained only 2.21 gm. of nitrogen. The relative toxicity of the two materials has not as yet been tested.

The loop shows an intact mucosa, which in general is pale, but shows a few indefinite areas of slight congestion. Microscopic sections show a normal mucosa which is covered with a dense gelatinous layer of material containing a few degenerated cells and nuclear particles, but for the most part is homogeneous, like gelatin. This is the thick, adherent loop material which is so rich in the toxic proteose. The villi and epithelial cells covering the villi are normal.

The preceding experiment (Dog 16-110, Table I) merits careful consideration for several reasons. Reference to the first proteose injections in this dog<sup>2</sup> shows the usual reaction to proteose and some evidence of immunity or tolerance to the second injection.

1 month later, after complete recovery, this dog was operated upon, and a closed loop of jejunum produced. It is well known that these dogs suffer a characteristic intoxication, and it has been shown also (1) that dogs previously injected with proteose are less susceptible to the acute intoxication of a closed intestinal loop. Dog 16-110 with a closed intestinal loop showed a chronic type of intoxication rather than the acute reaction usually seen in non-immune dogs, presumably due in part to the proteose injection 1 month before—compare the acute intoxication of the next experiment (Table II) in a non-immune dog.

The clinical signs of proteose intoxication in Dog 16-110 are slight or absent, and the curve of nitrogen elimination is of considerable in-



TABLE I.

*Dog 16-110. Long Closed Loop of Jejunum. Total Nitrogen Elimination. Immune to Proteose.*

Day.	Urine.		Feces nitrogen.	Weight.	Remarks.
	Total nitrogen.	Amount.			
	gm.	cc.	gm.	lbs.	
1	2.20	200	0	26.63	Fasting previous 3 days.
2	1.99	—	0.31*	26.13	
2	Long loop of jejunum isolated.				
3	2.35	150	0	26.13	Temperature 38.8°C.
4	4.23	—	0.17	—	Vomit in urine.
5	4.27	600	0	25.06	No vomitus.
6	3.56	600	0	24.44	Temperature 38.6°C.
7	2.86	455	0	24.19	“ 38.5° “ Wound swollen.
8	3.02	460	0	23.88	Clinically normal.
9	2.72	400	0	23.44	Wound firm.
10	2.38	515	0	23.31	
11	2.30	540	0	22.75	
12	2.44	410	0	22.56	
13	2.41	440	0	22.44	
14	2.20	565	0	22.19	
15	2.07	370	0	21.94	
16	2.13	460	0.39	21.81	
17	2.16	425	0	21.31	
18	2.24	450	0	21.25	Intestinal peristalsis visible.
19	1.99	450	0	20.88	
20	2.02	400	0	20.69	
21	1.90	405	0	20.56	
22	2.02	335	0	20.44	4 p.m. Fed 50 gm. of milk sugar, 100 gm. of vegetable fat (“Crisco”).
23	1.90	345	0	20.50	Fed milk sugar and fat.
24	1.68	295	0	20.50	
25	1.90	455	0	20.19	
26	2.24	355	0.14	20.0	4 p.m. Fed 15 gm. of milk sugar, 40 gm. of vegetable fat (“Crisco”).
27	2.10	390	0	19.81	
28	1.83	355	0.10	19.69	12 m. 100 gm. of milk sugar by stomach tube.
29	2.97*	—	±	19.44	Diarrhea.
30	1.88	465	0	19.06	4 p.m. 50 gm. of cane sugar by stomach tube.

\* Feces and cage washings.

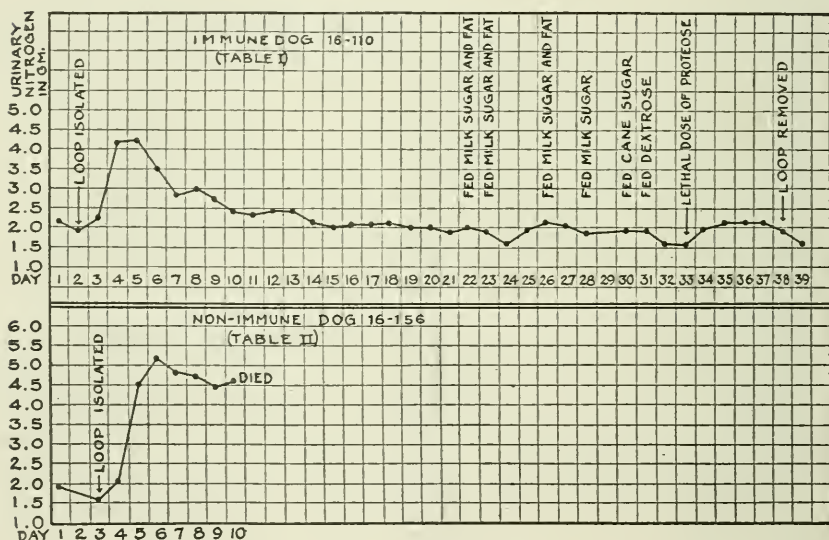
TABLE I—*Concluded.*

Day.	Urine.		Feces nitrogen.	Weight.	Remarks.
	Total nitrogen.	Amount.			
	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>lbs.</i>	
31	1.83	750	0	19.0	1 p.m. 50 gm. of dextrose by stomach tube.
32	1.65	740	0	18.94	
33	1.68	272	0	18.56	Visible peristalsis in abdomen.
33	Proteose from intestinal loop. Control dose fatal for normal dog.				
34	1.93	450	0.55	18.06	Temperature 38.1°C.
35	2.24	320	0	17.69	" 38.3° "
36	2.24	320	0	—	
37	2.21	335	0	17.44	
38	1.88	265	0	17.13	Temperature 38.2°C.
38	Closed loop of intestine resected. Loop contents total nitrogen 5.21 gm.				
39	1.64	160	—	16.19	Recovery.

terest. There is a sharp initial rise following the operation, but then a gradual fall toward the normal base-line elimination which is reached at the end of the 3rd week. A part of the initial rise is due to the wound reaction, and this is accurately controlled in Table VI, where the same dog after a considerable interval with recovery to normal was used for a control laparotomy experiment. The greater part of the rise in urinary nitrogen above the base-line (Text-fig. 1) is due to the intoxication associated with the closed loop of jejunum.

During this entire experiment, the dog was fasting. He returned to a normal or almost normal base-line nitrogen elimination during the 3rd week. There are no clinical signs of intoxication, but we note the presence of peristalsis in the abdomen—a sign that the loop is filling up. Sugar and fat feeding suggest an abnormal condition in this dog (Table I); they do not depress the curve of nitrogen elimination as in normal controls. There is not the same protein-sparing action. These experiments are merely suggestive, and more detailed work now in progress is required to clear up this point. It is possible that these experiments may throw light on the subject of protein-carbohydrate metabolism in disease.

Finally a lethal dose of proteose was given to this dog with little, if any, clinical reactions and a trifling rise in the curve of nitrogen elimination. This indicates a maximum tolerance for proteose which could scarcely arise except following a long continued chronic proteose intoxication. We assume that the presence of the closed intestinal loop was responsible for this chronic intoxication, which gave this dog such a high degree of tolerance to proteose injection. An occasional injection of proteose will give a certain degree of tol-



TEXT-FIG. 1. Closed loop of jejunum. Total nitrogen elimination of immune and non-immune dogs.

erance, as is seen in the first experiments on this dog, but this tolerance or immunity is relatively slight and transient (1), and cannot explain such a degree of protection as is noted after this injection of a lethal dose of proteose.

Table II (Dog 16-156) gives a good example of the nitrogen elimination and intoxication which may follow an experimental closed intestinal loop in a normal, non-resistant dog. It should be noted that the nitrogen elimination rises well over 100 per cent above normal,—almost 200 per cent above the normal base-line on 1 day. The

complicating wound infection was present only during the last 2 days, and may have helped a little in the acute intoxication which was more rapidly fatal than usual.

TABLE II.

*Dog 16-156 (Non-Immune). Long Closed Loop of Jejunum. Total Nitrogen Elimination.*

Day.	Urine.		Feces nitrogen.	Weight.	Remarks.
	Total nitrogen.	Amount.			
	gm.	cc.	gm.	lbs.	
1	1.85	40	0	13.75	Fasting previous 4 days.
2	1.29*	—	±	13.56	Some urine lost.
3	1.68	70	0	13.31	
3	Long loop of jejunum isolated.				
4	2.27	515	0.43*	12.44	Vomitus in urine. Temperature 39.2° C.
5	4.48	650	0	12.06	" " "
6	5.21	1,260	0	12.00	Some urine lost. Temperature 38.6° C.
7	4.84	235	0	11.81	Vomitus in urine.
8	4.76	150	0	11.50	" " "
9	4.45	125	0.89*	11.06	No vomitus. Infected wound.
10	4.64	130	0	10.75	" " Temperature 37.9° C.
11	1.13	80	0	—	Died during previous night.

\* Feces and cage washings. Loop contents total nitrogen 2.21 gm.

*Dog 16-156.*—(Table II.) Fox-terrier; adult male.

May 9, 1916. Metabolism experiment started (Table II).

May 12. Ether anesthesia and operation to produce the usual closed loop of intestine. The better part of the jejunum was isolated, the ends turned in, and the small intestine united around it by means of an end to end anastomosis. Time of operation, 1½ hours.

May 13-14. The dog shows a good deal of acute intoxication, and vomits repeatedly. Wound looks well.

May 15-16. Intoxication still marked.

May 18. Wound is somewhat infected, and the skin edges are broken down.

May 19. Dog is sick and temperature falling. Died in night. Autopsy next morning. The abdominal wound shows superficial infection, but the deeper layers are clean. The peritoneum is clean, but there is a slight excess of fluid.

Thorax, heart, and lungs are all normal. Liver and kidneys show cloudy swelling. Spleen is small. Urinary bladder is empty, and shows no inflammation. The entero-enterostomy is patent and well healed. The gastro-intestinal tract is normal.

The loop of jejunum measures 107 cm. in length. It is acutely distended and deep red. It contains 155 cc. of thick, slimy, reddish slate-colored fluid. The mucosa is reddish and velvety throughout. The ends of the loop are well closed. The loop fluid contained 2.21 gm. total nitrogen. The loop of intestine produced in this experiment (Table II) is similar to that of the preceding experiment (Table I). This dog (No. 16-156) was normal, and had received no proteose injections. Perhaps partly because of this he developed an acute intoxication, which rapidly led to a fatal termination. Table II contrasts the relatively acute intoxication with the more chronic poisoning noted in Table I in the dog which had been previously injected with proteose.

TABLE III.

*Dog 16-129. Duodenal Obstruction. Total Nitrogen Elimination.*

Day.	Urine.		Feces nitrogen.	Temper- ature.	Weight.	Remarks.
	Nitrogen.	Amount.				
	<i>gm.</i>	<i>cc.</i>	<i>gm.</i>	<i>°C.</i>	<i>lbs.</i>	
1	1.75	80	0	—	16.06	Fasting previous 4 days.
2	1.65	150	0	—	15.75	
3	1.79	80	0	—	15.62	
4	1.62	80	0.50	—	15.31	
4	Duodenal obstruction. Gastrojejunostomy.					
5	2.38	—*	0	38.4	14.44	Good recovery.
6	3.95	800*	0	38.5	14.06	Definite intoxication.
7	4.87	—*	0	37.9	13.75	Sick.
8	4.76	400*	0	37.3	13.44	
9	4.84	405*	0	—	13.0	
10	4.90	270*	0	37.5	12.56	Little vomitus.
11	3.44	165*	0	36.4	12.06	Subcutaneous abscess.
11	Killed. Autopsy performed at once. Blood non-protein nitrogen, 158 mg. per 100 cc.					

\* Vomitus and cage washings.

*Dog 16-129.*—(Table III.) Active, young fox-terrier; male.

Mar. 11, 1916. The dog had recovered from a mild attack of distemper. He was given a proteose injection of standard material prepared as usual from a closed loop of intestine. There were a moderate temperature reaction and some evidences of intoxication.

Apr. 8. The dog is in good condition (Table III).

Apr. 11. Ether anesthesia and operation as usual to produce duodenal obstruction. Time of operation, 1½ hours. The jejunum about 1 inch beyond



the duodenojejunal junction was cut across, and the upper end closed by inversion. The lower cut end was joined to the stomach, and a gastrojejunostomy performed as usual.

This dog (No. 16-129) shows an acute intoxication following this type of duodenal obstruction with gastrojejunostomy. The picture is somewhat complicated by an infected abdominal wound with an abscess developing just before death.

Apr. 18, 1 p.m. Dog is very sick. Ether anesthesia and bleeding from carotid.

The blood shows 158 mg. of non-protein nitrogen per 100 cc. and 70 mg. of urea nitrogen—or 44 per cent of total nitrogen.

There is an abscess in the subcutaneous tissue near the wound. It extends toward the costal margin, and contains 3 to 5 cc. of pus. There is little edema, and the edges of the wound are closed and well healed. The peritoneum is clean and moist. The thorax, heart, and lungs are normal. The liver, pancreas, spleen, kidneys, and bladder show nothing of importance. The stomach is slightly dilated, and contains bile-stained fluid. The gastrojejunostomy is patent, and the small intestine shows slight congestion only. The stomach shows several superficial erosions not over 0.5 cm. in diameter, one in the antrum and two close to the gastrojejunostomy. The duodenum is slightly dilated with bile-stained fluid. Its mucosa is intact but slightly congested. The lower end is well closed.

*Dog 16-136.*—(Table IV.) Black cocker spaniel; adult female.

Mar. 21, 1916. Splenectomy done as usual; rapid recovery.

Apr. 17. Experiment begun; the details are recorded in Table IV. Dog in normal condition. Fasting for previous 3 days.

Apr. 20. Ether anesthesia and operation as usual to produce a duodenal obstruction at the junction of the duodenum and jejunum with a gastrojejunostomy. The operation was identical with that in the preceding experiment (Dog 16-129); duration 1½ hours.

The clinical course of this animal is less acute than the preceding, and there are several days which show no vomitus, and give confirmatory data to show that the loss of nitrogenous material in the vomitus is slight, and can be neglected without introducing an appreciable error.

May 4, 2 p.m. Died. Autopsy performed at once. The serous cavities are negative. The parenchymatous organs show nothing of importance. The stomach and duodenum are somewhat distended, and show an intact mucosa. The gastrojejunostomy shows a large opening, and is efficient. The dog died from simple intoxication with no complicating factors except the splenectomy which will not be discussed at this time.

The two preceding experiments (Tables III and IV) deal with a type of duodenal obstruction which is of considerable value in experimental work. A simple obstruction at the duodenojejunal



TABLE IV.

*Dog 16-136. Duodenal Obstruction. Total Nitrogen Elimination.*

Day.	Urine.		Temper- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	<i>gm.</i>	<i>cc.</i>	<i>°C.</i>	<i>lbs.</i>	
1	1.98	100	—	22.75	Splenectomy previously.
2	1.65	110	—	22.56	
3	1.93	154	—	22.31	
3	Duodenal obstruction. Gastrojejunostomy.				
4	2.74	300	38.3	21.75	Good recovery.
5	2.98	800*	—	20.68	Vomit.
6	5.17	500*	—	20.75	Little vomitus.
7	4.62	180*	38.0	20.31	“ “
8	3.63	250*	—	20.06	“ “
9	3.64	150	—	19.81	No vomitus.
10	4.14	330*	37.9	19.38	Little vomitus.
11	3.19	300	38.3	19.19	No vomitus.
12	4.42	231	38.1	18.62	“ “
13	2.74	285	—	18.25	“ “
14	2.49	245*	38.1	17.94	Little vomitus.
15	3.30	240	37.3	17.56	A little feces.
16	3.33	250	36.8	17.25	No vomitus.
17	1.42	175	35.8	17.0	“ “
17	Died; autopsy.				

\* Vomitus and cage washings.

junction in a dog will lead to a fatal outcome usually in from 3 to 6 days with much vomiting and loss of fluid. The type of duodenal obstruction used here (Tables III and IV) gives a more chronic form of intoxication with little vomiting and loss of fluid—a condition much more desirable for experimental work. A complete obstruction of the duodenum is effected at its lower end by section and inversion of the intestine. The jejunum is joined to the stomach by a gastrojejunostomy, which allows fluid from the stomach to escape into the small intestine, and has much to do with the prevention of vomiting and the consequent loss of body fluids. There is present with this type of duodenal obstruction a definite intoxica-

tion, as we see from an examination of Tables III and IV. Under these conditions a dog may live from 1 to 3 weeks, and show little tendency to vomit, yet there are definite signs of intoxication, and such dogs can be shown to have a definite tolerance to proteose injections, as was shown in the case of closed loop experiments (Table I).

Both experiments show a great rise above the base-line of nitrogen elimination in the urine. The first experiment (Dog 16-129, Table III) shows a more acute course and a higher nitrogen curve. The second experiment (Dog 16-136, Table IV) shows a more chronic intoxication and a nitrogen curve which is quite high and sustained considerably above normal up to the end. The second experiment shows little loss of fluid by vomitus, and gives a pretty good control of this factor.

Tables V and VI give the necessary controls for the operative procedures employed in the preceding experiments. Similar experiments to control ether anesthesia and simple laparotomy have been done with identical results, and need not be reported.

TABLE V.

*Dog 17-10 (Normal). Laparotomy Control. Total Nitrogen Elimination.*

Day.	Urine.		Feces nitrogen.	Tempera- ture.	Weight.	Remarks.
	Total nitrogen.	Amount.				
	gm.	cc.	gm.	°C.	lbs.	
1	1.99	—	0	38.3	17.0	Fasting previous 4 days.
2	2.07	64	0	38.3	16.63	
3	1.95	60	0	38.3	16.44	
3	Laparotomy control (midline). Ether for 30 minutes.					
4	2.81	270	0	38.9	—	Little vomitus.
5	3.14	165	0	38.6	15.94	Wound edematous.
6	2.85	135	0	38.7	15.63	
7	2.52	90	0	38.6	15.38	Wound healing well.
8	2.32	70	0	38.7	15.31	
9	2.36	70	0	38.2	15.0	
10	2.50	100	0	38.1	14.75	Wound firm.
11	2.09	95	0	38.1	14.69	
12	1.96	92	0	38.3	14.38	Normal.

TABLE VI.

*Dog 16-110. Ether Anesthesia and Laparotomy Control. Total Nitrogen Elimination.*

Day.	Urine.		Feces nitrogen.	Temper- ature.	Weight.	Remarks.
	Total nitrogen.	Amount.				
	gm.	cc.	gm.	°C.	lbs.	
1	2.11	210	0	38.9	19.5	Fasting previous 10 days.
2	1.97	230	0	—	19.13	
3	1.75	188	0	38.2	19.06	
3	Surgical anesthesia. Ether for 1 hour.					
4	2.27	250	0	38.0	18.75	Good recovery.
5	1.69	195	0	37.9	18.25	
6	2.00	215	0	38.0	18.06	
7	1.89	220	0	37.9	17.94	
7	Laparotomy control. Rectus incision. Ether for 30 minutes.					
8	2.58	200	0	37.7	17.56	Good recovery.
9	2.55	365	0	—	17.50	Diuresis.
10	2.39	340	0	37.8	17.06	Wound slightly swollen.
11	2.23	275	0	37.8	16.88	
12	1.93	235	0.22	37.3	16.69	Wound firm.
13	2.00	270	0	37.7	16.44	Normal.

Ether anesthesia for 1 hour causes only a trivial rise in the curve of nitrogen elimination in the urine. In some cases the increase may be so slight as to be doubtful, and fall within the fluctuation due to the methods of collection and analysis.

Laparotomy was done in the usual manner, either in the midline or through the rectus. A few coils of intestine were brought out and handled as in preparation for an intestinal anastomosis. The incision was closed by silk and catgut, as in the other operations. The total increase in urinary nitrogen above the base-line after a laparotomy corresponds closely to the same rise noted after the injection of a single dose of toxic proteose.<sup>3</sup> We may assume with considerable certainty that the injured tissue which results from the

<sup>3</sup> See Table II of the preceding paper.

incision and closure by ligatures plus the slight bacterial infection of the clean wound can cause a suitable disintegration of tissue protein to form certain toxic split products which are responsible for this general reaction.

Histologically we may recall that a wound healing *per primam* is in reality a thin linear abscess with all the ingredients of the true abscess—tissue necrosis, skin bacteria, and wandering cells. There is no massing of cells sufficient to produce gross evidence of tissue and cell solution (pus), but we know that the injured cells go to pieces in the wound, and are removed by solution or phagocytosis. We may assume that this process is capable of forming various protein split products—probably among others a little toxic proteose which may be in part responsible for the general reaction and the increase in nitrogen elimination.

We may anticipate a report to appear in the near future dealing with various types of abscess, and say that this reaction produced by a clean wound is multiplied several times by an abscess reaction; but in all essentials the reactions are alike, the difference is merely quantitative. Further, it is possible to isolate toxic proteose-like substances from the pus of these abscesses.

#### DISCUSSION.

The experiments described above throw some light on the intoxication which appears in association with isolated intestinal loops or intestinal obstruction. There is a great increase in the urinary nitrogen above the fasting base-line level, and this rise in nitrogen elimination is in general proportional to the severity or acuteness of the intoxication. This great increase in urinary nitrogen indicates a considerable breaking down of body protein, and this destruction of protein is probably an important part of the intoxication. This explains in great measure the rise of non-protein nitrogen in the blood which has been observed in these conditions (2).

Tolerance or immunity to proteose intoxication develops in these experimental conditions (closed loops or intestinal obstruction) and with this tolerance we note a tendency for the nitrogen curve to fall toward normal (Table I). Dogs that have been injected repeatedly with sublethal doses of toxic proteose show less evidence of protein

injury and a lower curve of nitrogen elimination<sup>4</sup> than normal controls. These dogs are tolerant to proteose injections, and also are much more resistant to the intoxication following intestinal obstruction or closed intestinal loops.

In Tables V and VI there is evident a definite diuresis which deserves some consideration. This diuresis is observed too, after single injections of proteose, but is effectually masked by the vomitus in the obstruction experiments. These dogs had access to water at all times, and in the main the observations indicate simply an increased thirst. Perhaps we may say the tissues craved water. In order to determine whether there is actual retention of fluid during periods of acute intoxication and elimination of this excess during later periods, we must perform more carefully controlled intake experiments with sufficiently chronic intoxication so that there may be no tendency to vomit. We have submitted some evidence in the preceding paper to show that in acute proteose intoxication the body protein takes up water from the blood. If recovery takes place, we must assume that this excess water will be thrown out by the body cells. The thirst noted in these intoxications may be one sign that the proteins of the body need more water. This delicate balance between the cell protein or colloids and the water of the blood will require much more study before we have an intelligent understanding of a few of these important phenomena. The proteose intoxications, too, may offer a valuable means for further study of these phenomena.

Table I gives some data on the protein-sparing action of carbohydrates and fat in a chronic intoxication associated with an isolated intestinal loop. These few preliminary observations merely suggest that the protein-sparing action of carbohydrates and fat is not so definite in chronic proteose intoxications as in normal fasting dogs. We hope to report further on this point, and there are obviously many possibilities which will call for further experimental work. These conditions of proteose intoxication do not show any evidence of acidosis.<sup>5</sup>

<sup>4</sup> See Text-fig. 1 of the preceding paper.

<sup>5</sup> Certain of these experimental animals were included in a series of observations made by Dr. Whitney, and will be reported by him in detail in a separate publication.



## SUMMARY.

Dogs with isolated loops of small intestine show many evidences of intoxication. A study of the total nitrogen elimination shows a great rise above the normal base-line minimum of the fasting period (Table II). This means that the intoxication is associated with a great destruction of body protein, and explains the high non-protein nitrogen of the blood which was observed and reported previously (2).

Injection of a proteose obtained from a closed intestinal loop will cause a similar rise in the nitrogen elimination curve. This furnishes more evidence that the intoxication observed in association with a closed intestinal loop is in reality a proteose intoxication.

Dogs injected with sublethal doses of proteose will show a definite tolerance to subsequent injection, and will show much less acute intoxication after the isolation of a closed intestinal loop (Table I). These immune or tolerant dogs show a much less pronounced rise in the nitrogen elimination curve during proteose intoxication of any type. This indicates that the tolerance or immunity to proteose gives more protection for the body proteins against the injury which these toxic proteoses inflict upon the body cells.

Complete duodenal obstruction combined with a gastrojejunostomy gives a chronic type of intestinal obstruction associated with little vomiting, which is peculiarly suited to metabolism study (Table IV). Such duodenal obstructions show a definite and sustained rise in the curve of nitrogen elimination above the normal base-line level. These dogs, too, are tolerant to injections of standard toxic proteoses.

Control ether anesthesia experiments show little if any rise in the curve of nitrogen elimination (Table VI).

Control laparotomy experiments show a definite rise in the curve of nitrogen elimination, but a rise which is small compared with the rise noted in the intoxication of duodenal obstruction or of isolated intestinal loops. It is probable that the tissue injury and disintegration associated with the wound reaction are responsible for the general reaction. We may assume that protein split products from the wound area are absorbed and are responsible for the general reaction observed.

We propose to assume that the intoxications here studied are



associated with a definite proteose intoxication, which is capable of initiating and continuing a profound injury of tissue protein. One index of this protein injury is the great and sustained rise in the curve of total nitrogen elimination.

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2. Cooke, J. V., Rodenbaugh, F. H., and Whipple, G. H., *J. Exp. Med.*, 1916, xxiii, 717.

# THE EFFECT OF COLD UPON MALARIA PARASITES IN THE MOSQUITO HOST.

By W. V. KING, PH.D.

(From the Bureau of Entomology, U. S. Department of Agriculture, Washington, D. C., and the Laboratories of Clinical Medicine of the School of Medicine of Tulane University, New Orleans.)

PLATES 38 AND 39.

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The impression is gained from the literature on malaria that the development of malaria parasites in *Anopheles* is arrested at a temperature of about 60° F. and that the parasites themselves are destroyed at temperatures below this. The question is of importance since it involves the survival of infection in mosquitoes after hibernation or after exposure to low temperatures under natural conditions. In a series of experiments conducted in New Orleans from September to December, 1916, it has been found that the two common species of plasmodia, *Plasmodium vivax* and *Plasmodium falciparum*, in various stages of development in *Anopheles quadrimaculatus*, are able to survive exposure to lower degrees of temperature than 60° F. Furthermore, the results with *Plasmodium vivax* have shown that it may survive freezing temperatures of several days' duration,—a condition which rarely occurs naturally at this latitude.

In the present preliminary article the results which have furnished positive evidence of the resistance of *Plasmodium vivax* and *Plasmodium falciparum* to cold are presented. This evidence consists of the demonstration of the further development of oocysts and the viability of sporozoites subsequent to the exposure of infected mosquitoes to known and controlled temperatures varying from 60 to 29° F. and for periods of from 1 to 17 days.

The mosquitoes were bred specimens. They were infected by a single blood meal on suitable gamete carriers and were kept at room

temperature except for the periods of exposure. Controls from each lot of mosquitoes were kept under normal conditions for comparison with the experimental specimens.

The essential part of the data from experiments in which survival of the organisms was demonstrated is summarized in Table I.

TABLE I.

Experiment No.	No. of mosquitoes positive.	Time of development before exposure.	Temperature.	Duration of exposure.	Interval from exposure to dissection.	Evidence of resistance.				
						Subsequent development shown by.		Active sporozoites.		
						Oocysts.	Sporozoites in glands.	In oocysts.	In glands.	
Resistance of <i>Plasmodium vivax</i> in <i>Anopheles quadrimaculatus</i> .										
2,026	5	8	51-69 Mean 59.3	6		4-11	5	2	2	
2,027	3	8	30-31		24	3-12	3	1	1	
2,028	5	8	40		24	2-19	5	2	2	1
2,029	3	8	35		24	8-12	3	2	1	
2,030	2	7	30		24	5	8	2	2	
2,031	2	7	29-30		48	5	9-15		2	2
2,032	2	7	31		96	10	7-14	2	1	
2,033	1	14	31		94	25	2		1	
2,035.2	1	18	38-59 Mean 46.1	15	19		8			1
2,035.3	1	17	45-51 Mean 48	6	18		0		1	
2,035.6	1	15	38-59 Mean 46.1	16	20		13			1
2,037	1	23	29-30		49	30	1		1	
Resistance of <i>Plasmodium falciparum</i> in <i>Anopheles quadrimaculatus</i> .										
2,018	1	12	35		24		6			1
2,019	3	12	54-57		48		1-8			3
2,022	2	13	55-57		24		4-16			2

In Experiments 2,026, 2,027, 2,028, and 2,029 the infected mosquitoes were exposed on the 8th day after the infective blood meal. The stage of development is indicated by the microphotograph reproduced in Fig. 1. The mosquitoes in Experiments 2,030, 2,031, and

2,032 were exposed on the 7th day (see Fig. 2), but owing to the lower temperature of the room during growth, the oocysts were much less developed than were those in the previous experiments. In each of these series the recovery of the parasites was demonstrated in later dissections by conclusive evidence of further development of the oocysts. Mature cysts (Figs. 3, 4, and 5), sometimes with active sporozoites, and empty capsules after the release of the sporozoites, were found. In a number of specimens the salivary glands had become infected with sporozoites (Figs. 6 and 7), and in a few activity of these was observed. The mosquitoes in the remaining experiments were exposed when the parasites were in later stages of development, and in these the evidence of recovery was based upon the later finding of active sporozoites either in the glands or in oocysts from which they had not yet escaped (Fig. 8). Other specimens were not included in the positive results, since activity of the sporozoites was not determined, although they appeared normal.

#### SUMMARY.

The experiments reported here have shown that the parasite of tertian malaria in the mosquito host is able to survive exposure to a temperature of 30° F. for a period of 2 days, 31° F. for 4 days, and a mean temperature of 46° F. for 17 days. In a smaller series of tests the sporonts of the estivo-autumnal parasite have shown a resistance to temperatures as low as 35° F. for 24 hours.

I am indebted to Dr. C. C. Bass and Dr. F. M. Johns, of the Tulane Medical Department, for suggestions and aid. The mosquitoes were bred for the experiments at the Mound Laboratory of the Bureau of Entomology, in charge of Mr. D. L. Van Dine.

#### EXPLANATION OF PLATES.

##### PLATE 38.

FIG. 1. 8 day old oocysts in a control specimen from the lot of mosquitoes used in Experiments 2,026, 2,027, 2,028, and 2,029. Sporoblast formation was not yet distinguishable.  $\times 704$ .

FIG. 2. 6 day old oocyst in a control from the lot of mosquitoes used in Ex-

periments 2,030, 2,031, and 2,032. The exposure of the experimental mosquitoes was made a day later.  $\times 704$ .

FIG. 3. Four oocysts in final stages of development from a specimen in Experiment 2,028. (Compare with Fig. 1.)  $\times 704$ .

FIG. 4. An oocyst, with sporozoite development well advanced, from a specimen in Experiment 2,030. (Compare with Fig. 2.)  $\times 704$ .

PLATE 39.

FIG. 5. Several mature oocysts, from a second specimen in Experiment 2,030. (Compare with Fig. 2.)  $\times 704$ .

FIG. 6. Sporozoites from the salivary glands in a specimen from Experiment 2,031. (Compare with Fig. 2.)  $\times 330$ .

FIG. 7. Sporozoites from the salivary glands in a specimen from Experiment 2,026. (Compare with Fig. 1.)  $\times 330$ .

FIG. 8. An oocyst containing a mass of very active sporozoites 2 days after exposure to 31°F. for 94 hours, from a specimen in Experiment 2,033.  $\times 704$ .

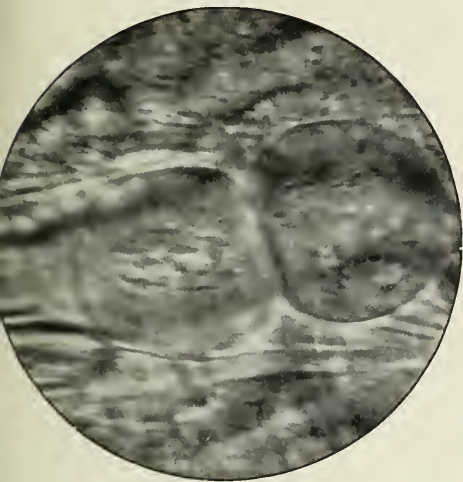


FIG. 1.

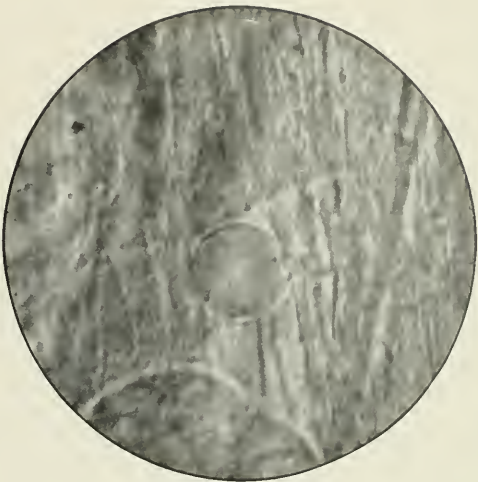


FIG. 2.

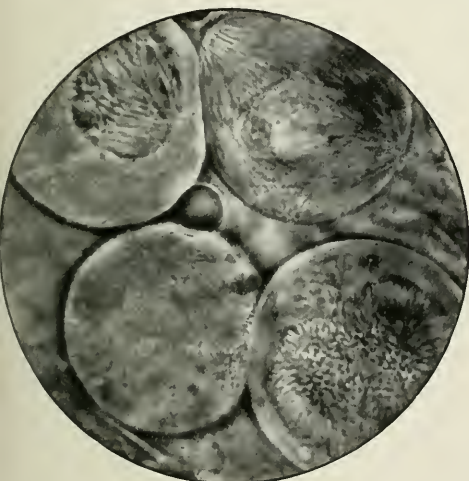


FIG. 3.



FIG. 4.

(King: Effect of Cold upon Malaria Parasites.)





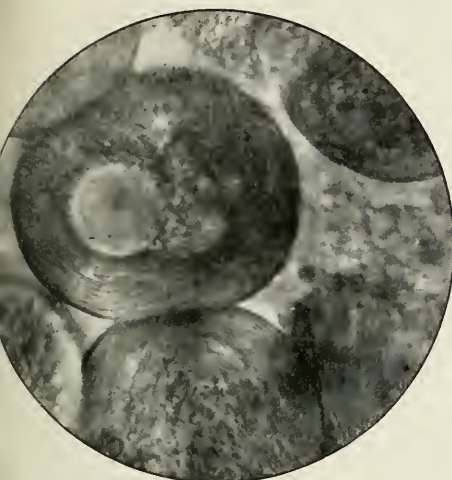


FIG. 5.

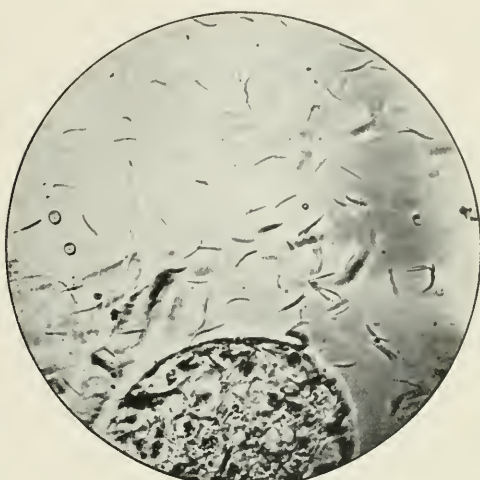


FIG. 6.



FIG. 7.



FIG. 8.

(King: Effect of Cold upon Malaria Parasites)



# THE PASSAGE OF NEUTRALIZING SUBSTANCES FROM THE BLOOD INTO THE CEREBROSPINAL FLUID IN POLIOMYELITIS.

BY SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Recovery in poliomyelitis as in other infectious diseases is accomplished through a process of active immunization. The immunity substances on which recovery depends have been detected by neutralization<sup>1</sup> and protection<sup>2</sup> tests, but not by other biological methods. Similarly, monkeys in which experimental poliomyelitis is induced by inoculation yield corresponding immunity bodies. Evidence exists to show that the neutralizing principles arise irrespective of the intensity of the clinical symptoms,<sup>3</sup> and apparently as early as the 6th day of the disease (page 501). Similar conditions exist in respect to the experimental disease in monkeys. Both protective and neutralizing principles have been discovered in animals which have passed through an attack induced by inoculation of filterable virus from human or from monkey sources.

The manner in which the immunity principles act in arresting the pathologic processes has become of especial interest on account of the treatment of cases of epidemic poliomyelitis by intraspinal injections of immune human and other sera. This method is based on the therapeutic experiments of Flexner and Lewis<sup>4</sup> with monkeys, the results of which were later extended to human beings by Netter and his coworkers<sup>5</sup> and since by many others.<sup>6,7,8</sup>

<sup>1</sup> Levaditi and Landsteiner, *Compt. rend. Soc. biol.*, 1910, lxviii, 311.

<sup>2</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 45.

<sup>3</sup> Netter, A., and Levaditi, C., *Compt. rend. Soc. biol.*, 1910, lxviii, 855.

<sup>4</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1780; lv, 662.

<sup>5</sup> Netter, A., Gendron, A., and Touraine, *Compt. rend. Soc. biol.*, 1911, lxx, 625.  
Netter, A., *Bull. Acad. méd.*, 1915, lxxiv, series 3, 403. Netter, A., and Salanier, M., *Bull. et mém. Soc. méd. hôp. Paris*, 1916, xl, series 3, 299.

<sup>6</sup> *Weekly Bull. Dept. Health, City of New York*, 1916, n. s. v, 345.

<sup>7</sup> Fischer, L., *Med. Rec.*, 1917, xci, 52.

<sup>8</sup> Amoss, H. L., and Chesney, A. M., *J. Exp. Med.*, 1917, xxv, 581.

Recently, however, both normal human serum<sup>6</sup> and normal horse serum<sup>9</sup> have been employed for intraspinal injection. It is still too early to state whether these latter sera exerted any definite influence on the pathologic processes.

All sera introduced into the subarachnoid spaces act as foreign bodies, and if sterile give rise to aseptic inflammation. The severity of the inflammation is less with homologous, than with heterologous serum. But experiments have shown that in monkeys very slight changes in the permeability of the meninges allow infection to take place after intravenous injection of the virus which otherwise is incapable of causing infection.<sup>10</sup>

Hence we have attempted to detect in the cerebrospinal fluid immunity principles such as exist in the blood. In the course of other experiments carried out by us<sup>11</sup> at a much earlier period, neutralization tests were made with the cerebrospinal fluid. Seven fluids were tested, of which only one proved neutralizing. This specimen came from a convalescent child paralyzed 6 weeks earlier. No examination was made at the time to discover whether the fluid indicated persistence of the inflammatory processes in the meninges. The conclusion reached was to the effect that, while possible, it was unusual for neutralizing principles to be contained in the cerebrospinal fluid during convalescence from epidemic poliomyelitis. Incidentally, it was determined that neutralizing bodies were not produced locally.

The present inquiry has arisen from the idea that under certain circumstances immunity substances enter the cerebrospinal fluid from the blood and assist materially in the healing process. If this supposition is founded on fact, we might view the inflammatory conditions occurring in the meninges, which increase their permeability to circulating proteins otherwise excluded,<sup>12</sup> as beneficial; and from this it may follow that any advantage actually shown to be derived from the intraspinal administration of normal human or horse serum

<sup>9</sup> Sophian, A., *J. Am. Med. Assn.*, 1916, lxvii, 426.

<sup>10</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.

<sup>11</sup> Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 205.

<sup>12</sup> Mott, F. W., *Lancet*, 1910, ii, 79. Flexner, S., *J. Am. Med. Assn.*, 1913, lxi, 447, 1872.

may be the result not of the effects of the serum as such, but of a further increase in this permeability.

*Neutralization Tests with Cerebrospinal Fluid.*

Three preliminary tests were made with human cerebrospinal fluid and one sample of human blood. Two of the former were drawn on the 2nd and 18th days, respectively, of the disease. One of them contained 920 cells per c. mm., and gave a positive test for globulin; the other contained 215 cells, and also gave a positive test for globulin. 2 cc. of each fluid were added to 0.1 cc. of filtrate of active monkey spinal cord, the mixture was incubated for 2 hours at 37°C., kept at 4°C. for 16 hours, and then inoculated intracerebrally, under ether anesthesia, into two *Macacus rhesus* monkeys. One monkey became paralyzed on the 6th, and the other on the 10th day. Both died. The third test with cerebrospinal fluid was made with an equal mixture of two specimens, one taken on the 6th, and the other on the 8th day of illness. Both contained an excess of cells and globulin. The inoculated monkey became paralyzed on the 7th day and afterwards developed contractures. It was etherized for histological study 6 weeks after inoculation. Hence none of the specimens tested contained demonstrable neutralizing substances.

The blood test was made with human serum obtained on the 6th day of illness. 1 cc. was mixed with 0.1 cc. of filtrate of active monkey virus, incubated, and kept as described above. The mixture was injected intracerebrally<sup>13</sup> into a *Macacus rhesus*. It remained well. Hence it appears that adequate neutralizing substances were present in the specimen.

The next experiment was made in order to determine whether the neutralizing substances may be made to pass from the blood into the cerebrospinal fluid. For this purpose normal monkeys were chosen. Each animal was given an intraspinal injection of normal horse serum and 16 hours later an intravenous injection of immune monkey serum from recovered poliomyelitic monkeys. The cerebrospinal fluid was withdrawn at different periods and tested for neutralizing properties. Every care was exercised to avoid admixture with blood. When

<sup>13</sup> Ether anesthesia was employed for the intracerebral inoculations.



blood appeared in the puncture needle, the fluid was discarded and the monkey yielding it was not used again for that experiment.

*Experiment 1.*—Dec. 5, 1916. 5 p.m. 2 cc. of normal horse serum were injected intraspinally into a *Macacus rhesus*. Dec. 6, 9.10 a.m. Injected 10 cc. of immune monkey serum intravenously. The cerebrospinal fluid was withdrawn 6, 9, and 24 hours later. The 6 and 9 hour specimens were combined in order to produce a total of 1 cc. Two neutralization tests were performed.

*Monkey A.*—Dec. 8. A *Macacus rhesus* received intracerebrally a mixture consisting of 0.1 cc. of active filtrate of poliomyelitic virus and 1 cc. of the combined 6 and 9 hour cerebrospinal fluids which had been incubated for 10 hours at 37°C. and kept for 3 hours at 4°C. No symptoms developed.

*Monkey B.*—Dec. 8. This test was repeated with the 24 hour specimen of cerebrospinal fluid. Dec. 18. Animal excitable and slow; protects right arm; ataxic. Dec. 19. Arms paralyzed; legs and back weak. Dec. 20. Prostrate. Dec. 21. Etherized.

Autopsy showed marked lesions of poliomyelitis in the spinal cord.

In these experiments the fluids withdrawn at the 6th and the 9th hours exerted a neutralizing effect, while the 24 hour specimen did not. The test was repeated as follows:

*Experiment 2.*—Jan. 23, 1917. 4 p.m. 2 cc. of normal horse serum were injected intraspinally into a *Macacus rhesus*. Jan. 24, 8.45 a.m. Injected intravenously 10 cc. of immune monkey serum. The cerebrospinal fluid was withdrawn 6 and 9 hours later and combined. One neutralization test was made with active filtered virus.

*Monkey C.*—Jan. 26. A *Macacus rhesus* received intracerebrally a mixture consisting of 0.1 cc. of filtrate virus and 1 cc. of combined spinal fluid incubated at 37°C. for 2 hours and kept at 4°C. for 15 hours. No symptoms developed.

This experiment confirms the previous one. On the other hand, in one test with fluid withdrawn at the 6th hour, neutralization was not effected. The protocol follows.

*Experiment 3.*—The procedure was identical with the two preceding experiments, except that the puncture was made at the end of 6 hours only. The cerebrospinal fluid was incubated with 0.1 cc. of filtrate virus and injected intracerebrally into a *Macacus rhesus* on Jan. 4, 1917. Jan. 13. Ataxia; widespread muscular weakness. Jan. 14. Prostrate; etherized.

At autopsy typical lesions of poliomyelitis were present.

. This series of experiments was controlled with the cerebrospinal fluid withdrawn from normal monkeys which had received an intraspinal injection of horse serum 18 hours before, but which had not received an intravenous injection of immune serum. An illustrative protocol follows.

*Experiment 4.*—Dec. 23, 1916. A *Macacus rhesus* was injected intracerebrally with an incubated mixture of 0.1 cc. of filtrate virus and 2 cc. of cerebrospinal fluid obtained from two monkeys which 18 hours before had received intraspinal injections of normal horse serum. Dec. 29. Tremor of head; ataxia; facial asymmetry; widespread muscular weakness. Dec. 30. Prostrate. Jan. 5, 1917. Etherized.

At autopsy typical lesions of poliomyelitis were present.

#### DISCUSSION.

The detection of the neutralizing immunity substances in the cerebrospinal fluid of human beings, at relatively early and later periods in the course of poliomyelitis, has been accomplished so rarely as to constitute a marked exception. At first sight, this observation would seem to indicate that the neutralizing principles do not pass into the fluid.

On the other hand, the experiments with passively immunized monkeys show unmistakably that the neutralizing substances are capable of passing into the cerebrospinal fluid under conditions in which the meninges have been inflamed. Later, however, when the inflammation is subsiding, the neutralizing substances either do not pass into the cerebrospinal fluid in detectable amounts or are fixed by the nervous tissues. However, cognizance must also be taken of the possibility in lightly passively immunized animals that the falling concentration of the immune bodies in the blood due to elimination may explain their failure to pass into the cerebrospinal fluid after the 9th hour.

While this point has not been settled, it is probable that in actively immune animals the passage of the neutralizing substances from the blood into the cerebrospinal fluid would continue as long as the inflammation present in the meninges rendered the structures easily permeable to the protein constituents of the blood. Probably the human fluids investigated by us were taken either at too early a period (2nd

day of the disease) or at too late a period (after the meningeal inflammation had subsided) to yield neutralizing effects. Until sufficient time has elapsed to permit the active immunization of the body to occur, no neutralizing substances are, of course, available. We have recorded an instance in which on the 6th day of illness the blood contained these substances in detectable amounts. Moreover, once the meningeal lesions are healed, these substances would no longer pass through. If, therefore, the healing process is inaugurated or facilitated by the passage of immunity substances from the blood into the cerebrospinal fluid, the height of that process would probably come in the first week of the illness.

What the experiments show conclusively is that once the neutralizing substances are contained within the blood, they can be made to pass into the cerebrospinal fluid by the production of an aseptic meningitis. We know that the injection of immune serum intraspinally tends to arrest the pathologic poliomyelitic process in monkeys and apparently are learning that it performs this purpose also in man. It is desirable, therefore, to consider what reason may exist for the therapeutic employment of non-immune sera, such as normal human and normal horse serum, in the treatment of cases of human poliomyelitis.

As far as present results can be interpreted, the therapeutic value of immune serum is largely confined to the early period of the disease corresponding with its onset.<sup>8</sup> The value is much less definitely shown, although not excluded, for the later stages when paralysis has already appeared and is extending. It is during the early period that the immunity processes are still in abeyance and that the blood is probably devoid of neutralizing substances. Hence no diversion into the cerebrospinal fluid could be accomplished at that time by increasing the permeability of the meninges through injections of normal serum. It is not easy to see what benefit may be expected at this most favorable time by the injection of normal serum, while it is obviously rational to employ an immune serum. Whether at later periods, when the immunity response has already taken place, the normal serum may hold out more promise cannot be stated; but as even immune serum is far less effective then, the promise seems to be small.

## CONCLUSIONS.

The cerebrospinal fluid taken very early and quite late in the course of acute poliomyelitis exhibits no neutralizing action on filtered poliomyelitic virus.

The blood serum on the 6th day of the disease already contains the neutralizing principles.

The injection of sterile horse serum into the cerebrospinal meninges in monkeys increases their permeability, so that they permit the immunity neutralizing principles passively injected into the blood to pass into the cerebrospinal fluid.

The passage in passively immunized monkeys takes place during a relatively brief space of time and apparently only while the inflammatory reaction produced by the horse serum is at its height.

It is established for monkeys and rendered probable for man that the intraspinal injection of immune serum in poliomyelitis is curative. In monkeys normal serum exerts no such action, and at present nothing can be stated definitely regarding the therapeutic effect of normal serum in man except that probably any benefits which may arise from its employment would be attributable not to the action of the serum as such, but to the escape of circulating immunity principles in the blood made possible by the aseptic inflammation set up by it in the meninges.

As the immunity principles appear in the blood only after several days, and the reported favorable effects of the immune serum treatment relate to the first days of illness, the employment of normal serum is thus not indicated, while that of an immune serum is.



## NEUTRALIZATION OF THE VIRUS OF POLIOMYELITIS BY NASAL WASHINGS.

BY HAROLD L. AMOSS, M.D., AND EDWARD TAYLOR, M.D.

(From the Research Laboratory\* of the Vermont State Board of Health, Burlington.)

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The occurrence of the infectious microorganism of poliomyelitis—the virus, so called—in the mucous membranes of the nasopharynx and in their secretions is now firmly established.<sup>1,2</sup> Not only may the virus be demonstrated by inoculation tests during the acute period<sup>3</sup> of the disease, but it is known to persist there in some cases for many months after convalescence<sup>4,5</sup> and, conversely, it has been detected in certain instances in the washings from the nasopharynx of healthy persons who have been in intimate contact with the acutely ill.<sup>6</sup> Finally, the fact has been determined by experiment that when the virus is introduced directly into the central nervous tissues wherein it multiplies, it also appears in the mucous membranes of the nose and throat. These facts indicate that the nasopharyngeal mucous membranes play an important part in the pathology of epidemic poliomyelitis; and the weight of opinion today is to the effect that the ingress and egress of the virus take place by way of these structures.

One of the most important questions arising out of the data presented above is that relating to the so called healthy carriers of the

\* Maintained by a special fund privately donated.

<sup>1</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 535.

<sup>2</sup> Landsteiner, K., Levaditi, C., and Pastia, C., *Semaine méd.*, 1911, xxxi, 296.

<sup>3</sup> Flexner, S., and Clark, P. F., *J. Am. Med. Assn.*, 1911, lvii, 1685. Landsteiner, K., Levaditi, and Danulesco, *Compt. rend. Soc. biol.*, 1911, lxxi, 558.

<sup>4</sup> Lucas, W. P., and Osgood, R. B., *J. Am. Med. Assn.*, 1913, lx, 1611.

<sup>5</sup> Kling, C., Pettersson, A., Wernstedt, W., and Josefson, A., *Communications Inst. méd. État à Stockholm*, 1912, iii.

<sup>6</sup> Flexner, S., Clark, P. F., and Fraser, F. R., *J. Am. Med. Assn.*, 1913, lx, 201. Kling, C., and Pettersson, A., *Deutsch. med. Woch.*, 1914, xl, 320.



virus, and for two main reasons. The healthy carriers may be the means of transporting the virus to other persons less resistant who may develop poliomyelitis; or the carrier, healthy when first contaminated, may subsequently develop the infection. At present the means at our disposal for studying the subject of virus carriers are so imperfect that no adequate notion of their number and distribution can be obtained. As long as the inoculation of monkeys with the washings from the nasopharynx must be relied upon to furnish this information, complete knowledge cannot be acquired.

There is, however, another fact which may prove to be significant. Assuming that during the prevalence of epidemics, many persons become contaminated with the virus, the question arises whether this condition need necessarily be either a menace to the contaminated person himself or to others. The answer to this question may lie in the reaction of the secretions of the nasopharyngeal mucous membranes to the virus present upon them. It is possible that in one person the secretions do not exercise a harmful action on the virus, while in another they do. This injurious action upon the virus may be of the nature of a protection to the individual contaminated as well as to the public in general.

It has often been observed that washings made from the nasopharynx may be ineffective when introduced into monkeys, and the lack of power to cause infection has been attributed to insufficient quantity or low infective power of the virus believed to be contained in the secretions removed. No note has been taken of the possibility that the washings are ineffective because the secretions of the mucous membrane are destructive or neutralizing to the virus of poliomyelitis. While this possible action may affect the inoculation tests in cases of acute poliomyelitis, it would be far more likely to be operative in the supposed carrier because of the small amount of virus and the probability of diminished virulence in the latter. Because of these considerations, a series of experiments was carried out to determine (1) the smallest quantity of a standard virus which can be detected in washings, and (2) the action of the washings of different persons upon the virus itself.

Reference has already been made to the fact that nasal washings, of contacts especially, have in a few instances produced polio-

myelitis when injected into monkeys. As the virus obtained directly from human beings possesses low virulence for monkeys, and is injected greatly diluted, the small number of successful inoculations is significant. Tests were made to determine the effect of concentration of washings on the activity of the virus. Amounts of virus which would certainly produce the infection if injected directly were added to a filtered washing fluid obtained from persons not having been exposed to the infection. The mixtures were separately reduced to small volume *in vacuo* at low temperatures and injected into monkeys. The results obtained were variable, for reasons which at first were not obvious, but the tests nevertheless showed that the filtered virus in certain amounts may withstand concentration in washing fluids without losing entirely its infective power.

#### EXPERIMENTAL.

*Experiment 1.*—The nasal cavities of a normal adult, H. L., were rinsed thoroughly with 50 cc. of distilled water. The collected fluids were passed through a Berkefeld filter and 0.2 cc. of a Berkefeld filtrate of a 5 per cent suspension of poliomyelitic brain was added. The mixture was reduced *in vacuo* at 37°C. to 4 cc. and injected intracranially, under ether anesthesia, into a *Macacus rhesus*. The monkey became partially paralyzed on the 10th day, completely prostrated on the 12th day, and died on the 14th day. Typical lesions of poliomyelitis were present.

*Experiment 2.*—The washings of a normal adult, W. T., were obtained in the manner described above. To the filtered fluid was added 0.5 cc. of a Berkefeld filtrate of active virus. The mixture was concentrated at 37°C. *in vacuo* and injected intracranially, under ether anesthesia, into a *Macacus rhesus*. The monkey remained well. As the result of a later protection test the monkey died of poliomyelitis after an appropriate injection of potent virus.

Filtration through Berkefeld or other porcelain filters is undertaken to remove the bacteria always present in the nasal and buccal secretions. But the bacteria can be either killed or their multiplication inhibited by certain antiseptic chemicals which affect to a less extent the virus of poliomyelitis. Thus 0.5 per cent carbolic acid destroys pyogenic bacteria in tissues and leaves the virus intact. Experiments also showed that ether acted more severely on the ordinary bacteria than on the virus. In order, therefore, to obviate any loss of virus which might result from its retention by the

filters, ether was employed to sterilize the washings. Preliminary tests showed that contact of ether for 20 hours with the virus contained in an emulsion of the spinal cord does not destroy it. The test made with washings indicates that while a shorter exposure may not kill all the bacteria, yet they are so greatly diminished that no ordinary infection is produced on inoculation into monkeys. And yet, as the experiments which follow show, while the filtrate contained in 0.8 per cent salt solution is active after the ether treatment, that mixed with the nasal washings is ineffective. The ineffectiveness at first believed to have been due to injury of the virus by the ether or too great dilution of the fluid inoculated, is now probably explicable in other ways.

*Experiment 3.*—1.8 cc. of a Berkefeld filtrate of active virus were added to 4.2 cc. of isotonic sodium chloride solution and 1 cc. of chemically pure ether. The mixture was shaken for 20 hours at room temperature. The ether was allowed to evaporate and 1 cc. of the remaining mixture, representing 0.3 cc. of virus filtrate, was injected intracranially, under ether anesthesia, into a *Macacus rhesus*. The monkey was almost prostrate on the 7th day, completely prostrate on the 8th, and etherized when moribund on the 10th day. The lesions were typical.

*Experiment 4.*—To 100 cc. of nasal washings from two normal adults was added 1 cc. of a Berkefeld filtrate of mixed virus, 0.1 cc. of which produced paralysis in the control monkey in 7 days. 5 cc. of chemically pure ether were added to the mixture and the whole was shaken for 20 hours at room temperature. The ether was allowed to evaporate, and 2 cc. of the mixture were injected intracerebrally and 98 cc. intraperitoneally under ether anesthesia into a *Macacus rhesus*. The monkey remained well.

Berkefeld filters withhold even very minute particles in greater amount when they are contained within a viscid or glutinous liquid. All the washings contain mucus; hence a procedure was adopted to modify the mucin so as to avoid this difficulty without at the same time injuring unduly the virus itself. The procedure consists in treating the washings with sodium bicarbonate, filtering, and then concentrating *in vacuo* at 37°C. The virus is little injured. When 0.1 cc. of a filtrate, which is on the limits of a minimum lethal dose, is used, the resulting concentrated fluid may be ineffective; when 0.2 to 0.3 cc. is employed infection results. The next protocol in which 0.3 cc. of filtrate was used is an example of the method, but identical effects were obtained with 0.2 cc.

*Experiment 5.*—The nasal cavity of a normal adult was thoroughly syringed with 50 cc. of sterile distilled water. 0.3 cc. of a Berkefeld filtrate of virus was added, and, after thorough mixing, 0.25 gm. of dry sodium bicarbonate was added and the fluid shaken for 20 minutes with beads. After centrifugation at high speed for 3 minutes the fluid was decanted and passed through a Berkefeld candle V. The precipitate was washed and filtered through the same candle. The mixture of the filtrates was reduced *in vacuo* at 36°C. to a volume of 2 cc., which with rinsing water was transferred to a collodion sac and dialyzed for 1 hour.

Under ether anesthesia a *Macacus rhesus* received half (3.5 cc.) of the resulting liquid into the left, and the other half into the right cerebral hemisphere. No symptoms were observed until the 6th day when the monkey became ataxic and excitable. The monkey was prostrate on the 8th day and died on the 14th day after injection. Typical microscopic lesions of poliomyelitis were present.

The treatment of the washings with sodium bicarbonate renders the effect of the inoculation certain when the use of larger quantities of the virus with washings alone fails to confer infection. The probable cause of the discrepancy has become apparent only after a more minute study of the properties of the nasal washings; but that the sodium bicarbonate acts either by allowing more virus to pass through the filter or by removing certain inhibitory influences exerted by the washings is directly indicated.

#### *Inactivating Effects of Nasal Secretions upon the Virus.*

The results of the preceding experiments, which contained obvious discrepancies, suggested a closer study of the secretions of the nose and pharynx from the standpoint of a possible inhibiting or neutralizing action on the virus of poliomyelitis. For this purpose a variety of persons was studied; some were suffering from acute poliomyelitis, and the others were apparently normal individuals.

The nasopharynx was rinsed with double distilled water and the washings were fractionally sterilized by heating to 60°C. for 3 successive days. Each person's specimen was handled separately. In earlier experiments, in order to economize animals, the washings of several persons were often mixed. It now seems not improbable that discordant results follow this procedure. The virus employed was obtained by filtering a 5 per cent suspension of glycerolated poliomyelitic monkey spinal cord. To each 30 cc. of the washing 7.5 cc. of the filtered virus were added. The mixture was then incubated at

37°C. for 24 hours. Control mixtures of virus and distilled water were subjected to the same incubation. Each cubic centimeter of the mixtures then contained 0.2 cc. of the filtrate, or at least two minimum lethal doses of the virus. The results of the first tests are given in Table I.

TABLE I.

*Inactivating Effects of Nasal Secretions upon the Virus.*

Date.	Virus No.	Dose of virus filtrate.	In contact with nasal washings from.	Method of sterilizing nasal washings before addition of virus.	Temperature at which virus, plus nasal washings were incubated for 24 hrs.	Result.
1916		cc.			°C.	
Feb. 16	32	0.2	Baby C.; age 3 yrs. Acute stage of poliomyelitis.	Heated 1 hr. at 60°C. on 3 successive days.	37	Monkey died. Typical poliomyelitic lesions.
Mar. 11	32	0.2	W. T., normal adult; age 39 yrs.	"	37	Monkey remained well.
June 2	48	0.2	C. A. R. and L. M. McK. (mixed), normal adults.	"	37	"
" 2	48	0.2	"	"	4	"
" 2	48	0.2	H. E. G., normal adult.	"	37	"

The results of this experiment suggest that the nasal washings of a person suffering from acute poliomyelitis may exercise no restraining influence upon an active virus, while those from healthy persons, under identical conditions of preparation, inhibit its activity.

The next experiment comprised tests on the nasal washings of eight apparently healthy persons. The results are recorded in Table II. At first sight it appears that of the eight specimens of washings, six possessed inhibiting properties and two did not. The question arose as to whether examination by a rhinologist, who would be unaware of the experiment, would disclose any differences in the nasal mucous membranes. These examinations, consented to by the



TABLE II.

*Inactivating Effects of Nasal Secretions of Adults.*

Date.	Dose of Berkefeld filtrate of Virus 48.	In contact with nasal washings from.	Method of sterilizing nasal washings before addition of virus.	Temperature at which virus plus nasal washings were incubated for 24 hrs.	Result.
1916	cc.			°C.	
Apr. 26	0.2	M. J. P., normal adult.	Heated 1 hr. at 60°C. on 3 successive days.	37	Monkey remained well.
" 26	0.2	E. S. S., " "	"	37	"
" 26	0.2	V. H. S., " "	"	37	"
" 26	0.2	C. A. R., " "	"	37	"
" 26	0.2	L. M. McK., normal adult.	"	37	"
" 26	0.2	G. H., normal adult.	"	37	"
" 26	0.2	J. P. B., " "	"	37	Monkey died. Typical lesions.
" 26	0.2	H. E. G., " "	"	37	"
" 26	0.2	Control (sterile water).		37	"
" 26	0.2	" (isotonic salt solution).		37	Monkey remained well.

persons, were kindly undertaken by Dr. M. C. Twitchell. His report is summarized in Table III. The only comment which the examination calls for is that while the anatomical condition of the nasal and adjacent mucosas in the six persons whose secretions contained inhibiting, inactivating, or neutralizing substances were normal, those of the other two were more or less pathologic. Just what the relation of this fact is to the effects of the secretions on the virus can only be surmised; but the test demonstrates that the secretions may frequently inhibit the action of the virus in monkeys. The control tests (Table II) show that, under the conditions of the experiments, distilled water injures the filtered virus less quickly than isotonic salt solution, a fact possibly dependent upon the different osmotic conditions present in the two fluids. The inactivation of



TABLE III.

*Results of Rhinoscopy of the Subjects Recorded in Table II.*

Case No	Age.	Report.
1 (M. J. P.)	23 <i>yrs.</i>	Normal nasal respiration; no discharge; no history of colds. Mild hypertrophic rhinitis. Septum deflected slightly to left with large horizontal ridge.
2 (E. S. S.)	24	Normal nasal respiration; no discharge; no throat symptoms; no history of colds. Mild hypertrophic rhinitis; vocal bands red (subacute laryngitis).
3 (V. H. S.)	24	Normal nasal respiration; no discharge; no throat symptoms; no history of colds. Mild hypertrophic rhinitis. Two spurs on left side of septum.
4 (C. A. R.)	20	Normal nasal respiration; no discharge; no throat symptoms; no history of colds. Mild hypertrophic rhinitis; septum deflected, half closes right nasal cavity.
5 (L. M. McK.)	22	Normal nasal respiration; no discharge; no throat symptoms; no history of colds. Mild hypertrophic rhinitis; small ulcer on left side of septum horizontal ridge on right side of septum.
(G. H.)	21	Normal nasal respiration; no discharge; no throat symptoms; no history of colds. Mild hypertrophic rhinitis. Acute pharyngitis of 3 days' duration.
7 (J. P. B.)*	22	Normal nasal respiration but easily obstructed when patient has a cold, especially left side of nose; secretion drops into throat on arising in the morning. Nose narrow; moderate hypertrophic rhinitis; large spur on right side of septum touches turbinate; small ulcer on left side of septum.
8 (H. E. G.)*	25	Nasal respiration interfered with, especially on right side; secretion drops into throat; frequent colds; has cold now. Septum deflected slightly to right; hypertrophic rhinitis; secretions found in right nasal cavity by anterior rhinoscopy. Acute pharyngitis and rhinitis.

\* Remarks by Dr. Twitchell: "No. 7 shows the most marked chronic nasal trouble of all, and I should class it as moderate rather than severe. No. 8, at the time of examination, had an acute rhinitis and an acute pharyngitis. This to a certain extent obscures the findings in this case. Frequent colds are a marked feature in the history of chronic rhinitis. No. 8 is the only one giving this history. I should conclude that if a chronic rhinitis produces changes in the nasal secretions, No. 8 would be the one whose nasal secretions were the most changed."

the virus through dilution by the washings and incubation at 37°C. would appear to be excluded by the results of the tests with the secretions and with the controls.

*Fluctuations in Inactivating Properties.*

Attempts were made to ascertain whether the action described is a constant or a variable property of the secretions. For this purpose washings were made at different times, sterilized by discontinuous heating at 60°C., and tested against 0.2 cc. of the filtrate which in control tests was determined to be potent. The results of these tests are given in Table IV.

TABLE IV.

*Fluctuations in Inactivating Properties.*

Date.	Case.	Condition.	Dose of virus filtrate.	Result.
1916			cc.	
Mar. 11	W. T.	Apparently normal.	0.2	Neutralized.
June 16	"	" "	0.5	"
July 12	"	" "	0.2	Failed to neutralize.
Nov. 14	"	" "	0.2	Neutralized.
Apr. 26	H. E. G.	Chronic rhinitis.	0.2	Failed to neutralize.
June 2	"	" " (improved).	0.2	Neutralized.
July 12	"	Apparently normal.	0.2	Failed to neutralize.
Apr. 26	C. A. R.	" "	0.2	Neutralized.
June 2	"	" "	0.2	"
Apr. 26	G. H.	" "	0.2	"
Dec. 18	"	" "	0.2	"
Apr. 26	L. M. McK.	" "	0.2	"
June 2	"	" "	0.2	"
Apr. 26	E. S. S.	" "	0.2	"
July 12	"	Acute coryza.	0.2	Failed to neutralize.

Of four tests with the secretions of W. T., three neutralized the virus; of three with those of H. E. G., only one neutralized it; of two with washings from C. A. R., G. H., and L. M. McK., respectively, all neutralized it, while in the case of E. S. S., one neutralized and the other did not. The animals that did not come down were subsequently determined to be susceptible to inoculation with the virus, so that the neutralization effects could not have been simulated by an excessive resistance on their part.

In addition to the tests described, which were conducted chiefly with adults, several were made with washings from children either healthy or suffering from poliomyelitis. The results are not wholly concordant. A larger series may possibly clear up the discrepancies.

Aug. 9, 1916. The washings of C. A., an apparently healthy boy, age 14, failed to neutralize 0.2 cc. of filtrate.

Oct. 23, 1916. The washings of R. J., age 8, taken during the acute attack of poliomyelitis, but after immune serum had been administered, neutralized 0.2 cc. of filtrate. A control monkey developed fatal, typical poliomyelitis.

Nov. 14, 1916. The washings of R. C., age 8, taken on the 15th day of the attack of poliomyelitis neutralized 0.2 cc. of filtrate. This patient had not been treated with immune serum. The control animal developed typical fatal poliomyelitis.

Feb. 16, 1916. The washings of B. C., age 3, taken during the acute stage of poliomyelitis did not neutralize the filtrate. Immune serum had not been given.

While the number of observations is too small to draw definite conclusions, it is obvious that the secretions of apparently normal persons vary in the so called neutralizing power. Of the two patients with poliomyelitis whose secretions inhibited action of the filtrate, one had received immune serum, while the washings were taken from the other on the 15th day, or at a time when immunity principles are known to be present in the blood.<sup>7</sup> The third child with poliomyelitis yielded washings without neutralizing effect; but they were taken earlier (4th day) in the course of the infection and at a time when the immunity bodies were probably not yet abundantly present. It is possible that some relation exists between the presence of definite immunity principles in the circulating blood and the power of the nasal washings to neutralize the virus.

In each series of experiments the potency of the virus was established by control experiments, and subsequently all the monkeys not showing symptoms were tested for immunity by appropriate injections of the virus and were all found to have been susceptible to infection. Hence the lack of response was not caused by an immunity of the animals employed. The secretions of three persons out of six examined varied in their power to neutralize 0.2 cc. of the virus filtrate at different times under nearly identical conditions,

<sup>7</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499

yet the only known clinical differences consisted in the presence of a rhinitis which appears to remove the inactivating power of the secretions.

*Fluctuations of the Inactivating Power in Abnormal Nasal Conditions.*

In April, 1916, the nasal secretions of E. S. S. neutralized 0.2 cc. of the virus filtrate, but 3 months later, during an attack of acute rhinitis, they did not. The washings from C. A. R. twice neutralized the same amount of virus at different times. Later, immediately following an acute rhinitis, no neutralizing power was observed, but the neutralizing power returned in 4 days.

The washings from H. E. G., taken when rhinoscopy revealed acute congestion of the nasal mucosa, did not possess neutralizing power, but 5 weeks later when the nasal condition had improved the washings showed the inactivating power. 6 weeks after the second test when there were no subjective symptoms of rhinitis, the washings failed to neutralize the virus. Finally, it will be recalled (Table III) that out of eight samples of nasal washings taken from apparently normal adults, only the two which were taken from subjects in which rhinoscopy revealed an acute rhinitis failed to inactivate the virus. H. E. G. is included in this list.

*Effect of Fractional Sterilization and Filtration on Inactivation.*

The experiments recorded indicate that the washings sterilized fractionally or passed through Berkefeld filters inactivate or neutralize virus mixed with them in the form of a filtrate of a suspension of the spinal cord of a poliomyelitic monkey. There can, therefore, be no doubt that the procedures do not themselves remove the neutralizing substances. Tests were then made to determine the comparative or quantitative effects of the procedures.

The quantity of filtrate employed for inoculation in this series of experiments was 0.4 cc., or more than four minimum lethal doses. The rinsings of the nasopharynx were made with redistilled water and they were reduced to a uniform volume of 15 cc. by concentration *in vacuo*. The fractional sterilization was carried out at 60°C. on 3 successive days. The washings and virus were left in contact 24

hours before the inoculations were made, in some instances at 37°C., in others at 4°C. The injections were intracerebral into *rhesus* monkeys under ether anesthesia. The results are given in Tables V and VI, and the comparison in Table VII.

The results lack absolute consistency. Considering the quantity of virus employed, the neutralizing action becomes more impressive. The variations in specimens from the same individual cannot now be accounted for. The existence of acute rhinitis, however, appears to diminish neutralizing power. Assuming that the process of neutralization is brought about by definite chemical bodies, they would seem to be thermolabile, since the neutralizing action of filtrates is definitely more pronounced than that of the heated specimens. Contact at 4°C. appears less effective in bringing about the neutralization than at 37°C. The prolongation of the incubation period noted in two instances is probably associated with partial but insufficient neutralization to reduce the virus below the minimum lethal dose.

### *Influence of Heat.*

The results given above suggest that the inactivating influence is weakened or destroyed by heat. The following experiment gives more definite information concerning this fact.

Washings were taken on Nov. 16, 1916, from W. T., whose nasal secretions had on several occasions proved neutralizing. 60 cc. of sterile distilled water were used and the washings passed through a Berkefeld N candle.

To 10 cc. of washings filtrate were added 2.5 cc. of active virus filtrate and the mixture was incubated at 37°C. for 24 hours. 1 cc. of the mixture, representing 0.2 cc. of the virus filtrate, was injected intracerebrally, under ether anesthesia, into a *Macacus rhesus*. The monkey remained well.

35 cc. of the washings filtrate were reduced quickly *in vacuo* at a temperature between 60° and 70°C. to a volume of 5 cc. 1.25 cc. of active virus filtrate were added and the mixture was incubated at 37°C. for 24 hours. 1 cc. of the mixture, representing 0.2 cc. of virus filtrate, was injected intracerebrally, under ether anesthesia, into a *Macacus rhesus*. The monkey was completely paralyzed on the 7th day and died on the 8th day. Typical lesions of poliomyelitis were present.

The neutralizing substance is apparently rendered inactive by heating to 70°C., though this experiment does not exclude volatility as the reason for the disappearance of this substance. Other experiments, however, in which the concentrations were carried out *in vacuo* at 60°C. indicate that the neutralizing substances are not volatile.

TABLE V.

*Neutralizing Power of Nasal Washings Heated to 60°C. for 1 Hour.*

Mon-key.	Dose of virus filtrate.	Fractionally sterilized nasal washings from.	Temperature at which virus plus nasal washings were incubated for 24 hrs.	Result.
	cc.		°C.	
A	0.4	W. T., normal adult.	37	Died in 33 days.
B	0.4	" " "	4	" " 31 "
C	0.4	G. H., " "	37	Remained well.
D	0.4	" " "	4	Died in 11 days.
E	0.4	C. A. R. (acute rhinitis).	37	" " 8 "
F	0.4	" " "	4	" " 8 "
G	0.4	Control (distilled water).	37	" " 14 "

TABLE VI.

*Neutralizing Power of Nasal Washings Passed through a Berkefeld Filter.*

Mon-key.	Dose of filtrate of virus.	Berkefeld filtered nasal washings from.	Temperature at which virus plus nasal washings were incubated for 24 hrs.	Result.
	cc.		°C.	
H	0.4	W. T., normal adult.	37	Remained well.
I	0.4	" " "	4	Died in 16 days.
J	0.4	G. H., " "	37	" " 19 "
K	0.4	" " "	4	Remained well.
L	0.4	C. A. R. (4 days after acute rhinitis).	37	" " "
M	0.4	" (4 " " " " ).	4	" " "



TABLE VII.

*Effect of Berkefeld Filtration and Heat on the Neutralizing Power of Nasal Washings*

Nasal washings from.	Condition of person from whom washings were obtained.	Result of neutralizing test against 0.4 cc. of Berkefeld filtrate of virus.			
		Filtered (Berkefeld) washings plus virus allowed to remain for 24 hrs.		Fractionally sterilized washings (60° C. on 3 days) plus virus allowed to remain for 24 hrs.	
		37° C.	4° C.	37° C.	4° C.
W. T., adult.	Normal.	+*	—	±†	±†
C. A. R., adult.	Acute rhinitis.			—	—
“ “	4 days after acute rhinitis.	+	+		
G. H., “	Normal.	—	+	+	—
Control (distilled water).				—	

\* The sign + indicates neutralization; ±, marked prolongation of the incubation period preceding paralysis.

† Incubation period greatly prolonged. Monkeys developed no symptoms until 33 and 31 days, respectively, after inoculation.

## DISCUSSION.

The power of the secretions of the nasopharynx of certain but not all individuals to bring about the inactivation or neutralization of the active virus of poliomyelitis has been demonstrated. The term active is employed to indicate that the samples of virus were obtained from strains adapted to the monkey, and could be relied upon to cause infection in the doses employed, almost without exception.

The inactivating property of the secretions mentioned is the more surprising in view of the resistance displayed by the poliomyelitic virus to such chemical antiseptics as glycerol and phenol.

In their manner of action, the neutralizing substances resemble more the specific immunity bodies contained within the blood serum of persons and monkeys who have suffered an attack of poliomyelitis. Like them, they appear to be thermolabile. And yet the experiments here recorded do not actually identify the two classes of substances.

It is known that the blood serum of certain adults who apparently

have never suffered from poliomyelitis is capable of neutralizing<sup>8</sup> the filtered poliomyelitic virus.<sup>9</sup> But in the few instances in which this property has been discovered, the adults yielding the serum had been in contact with acute cases of poliomyelitis, and artificial immunization cannot be excluded.

On the other hand, it seems not improbable that the inactivating or neutralizing power of the nasal secretions may play a part in protection against poliomyelitic infection, and even may represent an external system of defense against invasion of the virus by way of the nasopharyngeal mucosa.

If this view is supported by further studies, we should find that the secretions of children are less frequently neutralizing than those of adults, although many tests will be necessary to establish this distinction. In that case, we may find that the secretions of persons attacked by poliomyelitis at the period of onset of the disease lack neutralizing power, although later, when the immunization reactions have been aroused, inactivation may result, as has been shown to happen in particular instances in our series (page 516).

It appears, however, that the power of a given secretion to inactivate or neutralize the virus is not wholly a fixed one. Fluctuations in the property have been detected and described. Common and slight inflammatory conditions, *e.g.*, as in acute and even chronic rhinitis, apparently tend to remove or diminish the neutralizing power of the secretions. If this observation should be supported by further experiment, knowledge concerning one of the conditions favoring persistent contamination of the nasopharynx with the virus may be obtained. It does not follow, however, that this contamination need necessarily lead to infection, for the accomplishment of which disturbance of still other defensive mechanisms may be necessary. However, the production of healthy carriers of the poliomyelitic virus may rest upon the power or lack of power in the secretions to inactivate the virus. Should this be the case, then of many persons exposed only a fraction would become carriers, because the greater

<sup>8</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1780.

<sup>9</sup> Peabody, F. W., Draper, G., and Dochez, A. R., *A Clinical Study of Acute Poliomyelitis*, Monograph of The Rockefeller Institute for Medical Research, No. 4, 1912.

part would possess secretions capable of neutralizing and hence destroying the virus.

The variation in inactivating power does not depend alone upon inflammatory changes. Irregularities have been noted which cannot now be explained. They may be merely apparent and depend upon the experimental method to which we are at present limited. Inoculation experiments in single series are not wholly trustworthy. Filtration through porcelain is also open to errors of experiment, since the blocking of the porous spaces may easily exclude essential constituents of the washings. Fractional sterilization is also not a wholly reliable means of preventing bacterial development and yet of retaining unimpaired labile organic constituents. In view of all this, some degree of irregularity is to be looked for.

If this property of the secretions to inactivate or neutralize the virus of poliomyelitis is established, comparative tests should be made on large groups of persons at different seasons of the year in order to determine whether it bears any relation to the seasonal prevalence of poliomyelitis. We are engaged now in collecting observations covering this point; but reference to the tables will show that most of the tests were made during the spring, summer, and autumn. Moreover, they embraced few children of the most susceptible ages.

#### SUMMARY.

1. The results of 56 experiments have shown that washings of the nasal and pharyngeal mucosas possess definite power to inactivate or neutralize the active virus of poliomyelitis.

2. This power is not absolutely fixed, but is subject to fluctuation in a given person. Apparently inflammatory conditions of the upper air passages tend to remove or diminish the power of neutralization. But irregularities have been noted, even in the absence of these conditions.

3. Too few tests have been made thus far to ascertain whether adults and children differ with respect to the existence of this neutralizing property in the nasal secretions. While the inactivating property was absent from the secretions of one child during the first days of poliomyelitis, it was present in another to whom immune

serum was administered, and in still another on the 15th day of illness when convalescence was established.

4. The neutralizing substance is water-soluble and appears not to be inorganic; it appears to be more or less thermolabile, and its action does not depend upon the presence of mucin as such.

5. It is suggested that the production of healthy carriers through contamination with the virus of poliomyelitis may be determined by the presence or absence of this inactivating or neutralizing property in the secretions. Whether this effect operates to prevent actual invasion of the virus and production of infection can only be conjectured. Probably the property is merely accessory and not the essential element on which defense against infection rests. It is more probable that other factors exist which help to determine the issue of the delicate adjustment between contamination and infection.



## THE RELATION OF THE MENINGES AND CHOROID PLEXUS TO POLIOMYELITIC INFECTION.

By SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The prevention or promotion of an infectious disease is determined by the interaction of several factors. This may be assumed to be true of epidemic poliomyelitis. We know that the presence of the microorganism, or virus, of that disease upon the mucous membrane of the nose and throat does not necessarily lead to infection. This form of contamination apparently gives rise to a class of healthy carriers of the microbic cause of poliomyelitis. The prevailing view, based on analogy with other infectious diseases in which the portal of entry of the causative microorganism is the upper respiratory mucous membrane, notably epidemic meningitis, is to the effect that these carriers are several times as numerous as actual cases of infection. Obviously, therefore, mechanisms exist to protect the contaminated persons from the pathogenic action of the virus which they carry.

The incidence of poliomyelitis in communities in which it has prevailed has been low. Thus the case incidence of the Greater New York epidemic (1916) was 1.59 per 1,000 of the population.<sup>1</sup> From this fact a high degree of individual insusceptibility has been inferred,<sup>2</sup> and the basis of the condition has been sought in the notion that epidemic poliomyelitis is a far more common condition than has been supposed, and in very mild form may prevail extensively without being recognized as that disease.<sup>3</sup> Since even a slight attack leaves an enduring protection,<sup>4</sup> a state of general immunity has been created

<sup>1</sup> Emerson, H., *Am. J. Med. Sc.*, 1917, cliii, 160.

<sup>2</sup> Flexner, S., *J. Am. Med. Assn.*, 1916, lxxvii, 279.

<sup>3</sup> Wernstedt, W., *Communications Inst. méd. État à Stockholm*, 1912, iii, 235.

<sup>4</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 45; *J. Exp. Med.*, 1910, xii, 227.



which manifests itself later during severe epidemics attended with paralysis and high mortality. While our present knowledge does not permit a final statement as to the applicability of this hypothesis even to Northern European countries in which poliomyelitis has been endemic for the past 30 years, it seems entirely inapplicable to America and the other countries which have only recently come widely under the influence of the epidemic disease.

The discovery of the main portals by which the virus enters the body has focused attention on the conditions which favor or hinder its entrance.<sup>5,6</sup> Having reached the upper respiratory mucosa the virus may take either of two routes in invading the central nervous organs. It may penetrate first into the local blood vessels and be carried into the circulation and thence to the nervous tissues, or it may pass into the lymphatic vessels surrounding the olfactory nerves and ascend more directly to the brain, medulla, and spinal cord.

Experimental evidence suggests the latter route.<sup>7</sup> It is difficult to infect monkeys with highly active poliomyelitic virus by way of the blood; and, conversely, it is easier to infect them by way of the nasal mucosa.<sup>6</sup> It is the function of the choroid plexus and the pial lymphatic vessels to exclude the virus present in the blood from the nervous tissues. Once these protective structures are injured, the exclusion ceases and infection can be made to follow readily. Flexner and Amoss<sup>8</sup> found that the injection of normal horse serum into the meninges, through which a transient aseptic meningitis is produced, is an effective way of overcoming the obstacle interposed by the structures mentioned. Intactness or permeability may conceivably play a part in determining whether infection is to ensue or not. When the virus enters directly from the nasal mucosa to the brain, the medulla and lastly the spinal cord become infected,<sup>7</sup> from which it would appear that the virus entering the central nervous organs by way of

<sup>5</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 535.

<sup>6</sup> Levaditi, C., and Landsteiner, K., *Compt. rend. Soc. biol.*, 1910, lxxviii, 417. Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1140.

<sup>7</sup> Flexner, S., and Clark, P. F., *Proc. Soc. Exp. Biol. and Med.*, 1912-13, x, 1. Flexner, *Lancet*, 1912, ii, 1271; *Science*, 1912, xxxvi, 685; *Brit. Med. J.*, 1912, ii, 1261.

<sup>8</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

the olfactory nerves permeates the organs continuously and is not distributed by the general circulation.

In discussing the defensive mechanisms, it is desirable in the first place to consider the ultimate fate of the virus which reaches the nasal mucosa as it does in many more persons than develop poliomyelitis. Amoss and Taylor<sup>9</sup> have found that the secretions of the mucous membrane are capable of neutralizing or inactivating the virus; and their experiments show also that this neutralizing property is absent altogether from the secretions of some persons and fluctuates in those of others, being present at one time and not at another. We may, indeed, view this destructive power as a means by which the number of persons becoming contaminated with the virus in the course of epidemics is diminished, as well as a mechanism through which infection may actually be prevented. Hence it may constitute one factor in the complex state to which the term susceptibility is applied. That still other factors enter into the perfection of this state is indicated by the experiments to be described.

#### *Meningeal Disturbances and Intravenous Inoculation.*

The routes through which infection with the poliomyelitic virus may be accomplished in monkeys have been closely studied. In order of ease and constancy they are: the intracerebral, intranasal, intraperitoneal, intraneural,<sup>10</sup> subcutaneous, and intravenous routes. The virus may also enter by other and extraordinary channels which have been little studied; namely, after intraocular injection<sup>11, 8</sup> and after feeding in animals narcotized with opium.<sup>12</sup> The intracerebral route gives not only the most constant results, but it exceeds the others in delicacy of response. This fact has been emphasized in a previous paper as has the power of the virus to be augmented after an intravenous inoculation through an aseptic meningitis induced by the intraspinal injection of normal horse serum.<sup>8</sup> Since then we have

<sup>9</sup> Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

<sup>10</sup> Some authors regard intrasciatic inoculation as next to intracerebral in effectiveness; in our experience it constitutes an uncertain portal of entry of the virus.

<sup>11</sup> Landsteiner and Levaditi, *Compt. rend. Soc. biol.*, 1909, lxxvii, 787.

<sup>12</sup> Leiner, C., and von Wiesner, R., *Wien. klin. Woch.*, 1910, xxiii, 91.

ascertained that an intranasal application of the virus, otherwise ineffective, may be made infectious by a similar aseptic inflammation of the meninges. The meningeal mechanism, which includes the choroid plexus, has indeed proved to be not only determinative in respect to the effect of an inoculation of the virus, but also of remarkable delicacy of adjustment. Pathologic changes of almost incredibly slight character may set aside its protective function.

*Horse Serum.*—The intraspinal injection of normal horse serum brings about in monkeys profound changes in the meningeal mechanism.<sup>8</sup> Besides producing a rich cellular, protein exudate, the epithelial coverings of the choroid plexus are rendered more permeable. Under these circumstances an otherwise ineffective quantity of the virus introduced into the circulation becomes infectious. But even so the quantity of virus effective by way of the circulation exceeds considerably that necessary to produce infection by intracerebral injection.

*Experiment 1 (Control).*—Dec. 13, 1916. A *Macacus rhesus* received an intravenous injection of 50 cc. of a centrifuged 5 per cent suspension of active monkey spinal cord and medulla,<sup>13</sup> 0.1 cc. of a Berkefeld filtrate of which was infectious by intracerebral inoculation. The monkey remained well.

*Experiment 2.*—Dec. 21, 1916. 4 p.m. 2 cc. of normal horse serum were injected intraspinally into a *Macacus rhesus*. Dec. 22, 11 a.m. 1 cc. of centrifuged virus as in Experiment 1 was injected intravenously. The monkey remained well.

*Experiment 3.*—The same as Experiment 2, with 5 cc. of the centrifuged virus. The monkey remained well.

*Experiment 4.*—The same as Experiments 2 and 3, with 10 cc. of the centrifuged virus. Paralysis on the 6th day and death on the 9th day following the intravenous injection.

The greater efficacy of the intracerebral route of inoculation is brought out by the experiments. The discrepancy is greater even than it appears to be because the filtration of the centrifuged suspension removes a large part of the protein content and with it, of course, much, and perhaps most of the virus.

In the past the intracerebral mode of inoculation has been relied

<sup>13</sup> In order to avoid immediate toxic effects, the emulsion must be made from nervous organs freshly removed from the paralyzed monkeys. Specimens kept at 4°C. or in 50 per cent glycerol are not satisfactory. Their injection is often followed in a short time by a drop in blood pressure and respiratory failure.

upon in therapeutic and disinfection tests with the poliomyelitic virus because it alone gave constant results. This was a disadvantage since, in the first place, the reaction of monkeys to an intracerebral inoculation of the adapted virus is far more severe than the response of human beings to the infection in the ordinary way. With the active virus employed in this manner, the mortality among the inoculated monkeys is approximately 100 per cent. In the next place the choice of disinfecting agents was necessarily limited to those which could be introduced into the cerebrum along with the virus. These disadvantages have been overcome through the promoting action of horse serum and other agents, some of which were hitherto regarded as indifferent, introduced into the meninges.

*Normal Monkey Serum.*—An homologous serum injected into the meninges sets up an aseptic meningitis, of lighter grade than that induced by a heterologous serum. Normal monkey serum was tested for its power to promote infection following an intravenous injection of the poliomyelitic virus.

*Experiment 5.*—Dec. 12, 1916. A *Macacus rhesus* received an intraspinal injection of 2 cc. of clear, hemoglobin-free normal monkey serum. Dec. 13. Intravenous injection of 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla. Dec. 18. Animal moves somewhat stiffly; ataxia; tremor of head; ptosis; shoulder muscles weak. Dec. 19. Died.

The autopsy showed typical lesions of poliomyelitis.

As this experiment shows, normal monkey serum resembles normal horse serum in promoting infection from an intravenous injection of the poliomyelitic virus. Experiments 6, 7, 8, and 9 indicate that even a less degree of inflammatory reaction than that induced by normal serum suffices to open the way for the virus present in the blood to reach the nervous organs.

*Salt, Ringer's, and Locke's Solutions.*—Of the three fluids mentioned, those which physiologically are most indifferent are Ringer's and Locke's solutions. As far as visible responses are concerned, all produce less inflammation of the meninges than either horse serum or monkey serum. And yet they are clearly proved by the reactions to be foreign and in a degree injurious to the meningeal structures.

*Experiment 6.*—Dec. 19, 1916. 5 p.m. 2 cc. of sterile isotonic salt solution were injected intraspinally into a *Macacus rhesus*. Dec. 20, 11 a.m. 50 cc. of a centri-

fused 5 per cent suspension of active spinal cord and medulla were injected intravenously. Dec. 26. Ataxia; tremor of head; double ptosis. Dec. 27. Died.

The autopsy showed typical and marked lesions of poliomyelitis.

*Experiment 7.*—Dec. 19, 1916. 5 p.m. 2 cc. of sterile Ringer's solution were injected intraspinally into a *Macacus rhesus*. Dec. 20, 11 a.m. 50 cc. of centrifuged fluid similar to that in Experiment 6 were injected intravenously. Dec. 29. Ataxia; tremor of head. Dec. 30. Shoulder and back muscles weak. Jan. 2, 1917. Paralysis extended. Died.

The autopsy showed typical lesions of poliomyelitis.

*Experiment 8.*—*Macacus rhesus*. This experiment was exactly like the preceding one, except that sterile Locke's solution was used. Jan. 5, 1917. Intraspinal injection of Locke's solution. Jan. 11. The first symptoms appeared. Jan. 15. Died.

The autopsy showed typical poliomyelitic lesions.

In the next experiment, Locke's solution plus 0.5 per cent gelatin were employed.<sup>14</sup>

*Experiment 9.*—Jan. 23, 1917. 4 p.m. 2 cc. of sterile Locke's solution containing 0.5 per cent gelatin were injected intraspinally into a *Macacus rhesus*. Jan. 24, 10 a.m. 50 cc. of a centrifuged 5 per cent suspension of spinal cord and medulla were injected intravenously. Feb. 2. Paralysis of legs; shoulders weak. Feb. 4. Prostrate. Feb. 5. Died.

The autopsy showed typical lesions of poliomyelitis.

It is obvious that even when the irritative and inflammatory changes induced in the meningeal structures are actually very slight, they suffice to remove the power of the intact organs to exclude the virus from the interstices of the central nervous tissues. These observations, in themselves important, unexpectedly confirm and extend an earlier experiment in which the injection of salt solution into a cerebral hemisphere promoted infection with a virus, otherwise ineffective by that route, introduced into the veins.<sup>8</sup>

#### *Homologous and Autologous Cerebrospinal Fluid and Simple Lumbar Puncture.*

Since it now appeared that very slight changes in the meningeal structures enabled the virus circulating in the blood to penetrate and

<sup>14</sup> Rous and Turner (*J. Exp. Med.*, 1916, xxiii, 219) observed that a fluid of that composition was an excellent medium for preserving red corpuscles intact for a considerable period and surpassed in that respect the other artificial physiologically balanced fluids tested.



multiply within the central nervous organs, the question arose as to the limit of the irritative process sufficing for this purpose. The next experiments related to (1) simple lumbar puncture, (2) the removal and return of the cerebrospinal fluid in the same monkey, and (3) the withdrawal of the fluid from one monkey and its replacement with the fluid taken from other monkeys.

*Simple Lumbar Puncture.*—By this term is meant the removal of fluid by lumbar puncture under conditions in which no blood whatever is drawn by the operation. If a trace of blood entered the needle, the animal was not regarded as suitable for the test.

*Experiment 10.*—Dec. 21, 1916. 4 p.m. 2 cc. of clear fluid were withdrawn from a *Macacus rhesus* by lumbar puncture. Dec. 22, 12 noon. Intravenous injection of 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla. No symptoms developed.

*Withdrawal and Return of Autologous Fluid.*—In this experiment, as in the preceding one, the merest tinge of blood in the withdrawn fluid renders the animal unsuitable.

*Experiment 11.*—Jan. 15, 1917. 4 p.m. 2 cc. of clear fluid were withdrawn from a *Macacus rhesus* by lumbar puncture. 5 p.m. The same fluid was returned by lumbar puncture. Jan. 16, 10.30 a.m. Intravenous injection of 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla. No symptoms developed.

*Introduction of Homologous Fluid.*—The same precautions were followed in this experiment to avoid the slightest hemorrhage in performing the lumbar puncture.

*Experiment 12.*—Jan. 5, 1917. 6 p.m. 2 cc. of clear cerebrospinal fluid were withdrawn from a *Macacus rhesus* by lumbar puncture and replaced by 2 cc. of clear fluid withdrawn 3 minutes earlier from a normal monkey of the same species. Jan. 6, 11 a.m. Intravenous injection of 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla. Jan. 14. Legs, arm, and back weak. Jan. 15. Prostrate. Feb. 2. Died.

The autopsy showed characteristic lesions of poliomyelitis.

*Experiment 13.*—Feb. 8, 1917. 4 p.m. 2 cc. of clear cerebrospinal fluid removed 30 minutes before from two normal *Macacus rhesus* monkeys were injected intraspinally into a monkey of the same species. Feb. 9, 10 a.m. Intravenous injection of 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla. Feb. 14. Paralysis of legs. Feb. 15. Prostrate.

The autopsy showed typical lesions of poliomyelitis.



This experiment has not succeeded in every instance.

That the production of even slight hemorrhage in withdrawing the cerebrospinal fluid may seriously alter the result is shown by the next experiment.

*Experiment 14.*—Feb. 15, 1917. 4 p.m. 1 cc. of cerebrospinal fluid slightly tinged with blood was withdrawn from a *Macacus rhesus* by lumbar puncture. The quantity of blood present in the fluid, estimated on the basis of red corpuscles present, was perhaps one-fiftieth of the total volume of the fluid. Feb. 16, 10 a.m. Intravenous injection of 50 cc. of a centrifuged 5 per cent suspension of active virus. Feb. 20, a.m. Ataxia; tonic convulsions. p.m. Prostrate. Feb. 21. Died.

The autopsy showed typical lesions of poliomyelitis.

The preceding experiments indicate that (1) simple uncomplicated lumbar puncture does not lead to infection after an intravenous inoculation; (2) the simple removal and return of the same cerebrospinal fluid in a given animal is likewise without effect; while (3) the interchange of fluid from one animal to another does lead to infection, although not in every instance. Moreover, injury to the blood vessels of the meninges also promotes the infection. The infection in the last example may be due either to the escape directly of the inoculated virus from the blood through the injured vessels into the meninges, or what is more probable, to the blood's entering the subarachnoid space and setting up a mild inflammatory reaction, which, as we have seen, suffices to promote the infection. Sometimes the interchange of fluids fails to be followed by an infection. This failure may be due to one of several causes: the two fluids interchanged may be essentially so alike in composition as to constitute practical identity; the test then becomes equivalent to the autologous experiment; or the meningeal structures in certain monkeys may be less responsive to such a mild irritant and resist its action altogether.

It is more remarkable that the irritative response should occur than that it should in some instances fail to take place. The experiments emphasize the extraordinary sensitiveness of the meningeal protective adjustment through which exclusion of the poliomyelitic virus, and doubtless other injurious substances, from the interior of the nervous organs, is accomplished. They suggest that if alterations as slight as these suffice to produce infection, the essential meningeal mechanism may have to be regarded as in no small part determinative of the development of poliomyelitic disease.

*Serum Promotion of Intranasal and Subcutaneous Infection.*

Following Flexner and Lewis's<sup>5</sup> demonstration that the virus of poliomyelitis introduced into the cerebrum escapes by way of the nasal mucosa, Levaditi and Landsteiner<sup>6</sup> first determined that the application of the virus to the nasal mucosa would lead to infection. As already stated, the intranasal method is a less constant means of producing infection than the intracerebral and requires somewhat greater quantities of the virus.

In the following experiments inoculation was accomplished by packing the left naris with a plug of absorbent cotton saturated with crushed spinal cord of a recently paralyzed monkey. The plug was left in position for 2 hours and then removed, and not infrequently it showed slight tingeing with blood.

We have found that packing for 2 hours is insufficient to lead to infection in normal monkeys. But when monkeys have previously received an intraspinal injection of horse serum, infection results.

*Experiment 15.*—Jan. 2, 1917. 5 p.m. 2 cc. of normal horse serum were injected intraspinally into a *Macacus rhesus*. Jan. 3, 11 a.m. A pledget of cotton soaked with fresh poliomyelitic spinal cord of a monkey was packed in the left naris. When removed 2 hours later it was slightly stained with blood. Jan. 9. Protects right arm. Jan. 11. Tremor of head; ataxia; considerable spasticity of legs and weakness of deltoids. Cerebrospinal fluid contains 2,680 cells per c.mm. and increased globulin. Jan. 12. Prostrate. Jan. 14. Died.

The autopsy showed characteristic lesions of poliomyelitis.

*Experiment 16.*—Feb. 8, 1917. 4 p.m. A *Macacus rhesus* received an intraspinal injection of 2 cc. of normal horse serum. Feb. 9, 10.30 a.m. A nasal plug saturated with poliomyelitic spinal cord and medulla was introduced and left in the left naris for 2 hours. Feb. 18. Paralysis in left arm; weakness of back. Feb. 19. Prostrate. Feb. 20. Etherized.

The autopsy showed typical lesions of poliomyelitis.

The same method of promoting infection was used in the instances of intrasciatic and of subcutaneous inoculation. In two trials no result was accomplished in the former; while in the latter infection took place.

*Experiment 17.*—Jan. 9, 1917. 5 p.m. 2 cc. of normal horse serum were injected intraspinally into a *Macacus rhesus*. Jan. 10, 2 p.m. Subcutaneous injection of 1 cc. of a 5 per cent suspension of spinal cord and medulla of a poliomye-

litic monkey. Jan. 20. Arms paralyzed; back weak; facial asymmetry. Jan. 21. Died.

The autopsy showed typical lesions of poliomyelitis.

A control animal in which no horse serum had been inoculated did not respond to the subcutaneous inoculation alone.

This series of experiments indicates that preexisting pathologic conditions in the meningeal structures promote infection in poliomyelitis not only when the virus is present in the blood, but also when it reaches the central nervous organs by way of the nerves, as in the case of intranasal inoculation and probably also of subcutaneous inoculation. The promoting influence of the serum in intranasal inoculation acquires special significance in view of the fact that the nasal mucosa is the usual portal of entry of the virus into the nervous organs in human beings;<sup>15</sup> that is, the intact meninges are a protection from nasal as from intravenous inoculation with the virus of poliomyelitis, as, conversely, the pathologically altered meninges are a cause of heightened susceptibility to the action of the virus present in either place.

#### *Immune Serum and Heightened Susceptibility.*

The experiments described show that not only normal horse and monkey serum, but fluids of far less irritative nature promote, when introduced into the meninges, infection with the virus of poliomyelitis. Earlier experiments, on the other hand, have shown that infection with the virus may be prevented by an immune serum even when the inoculation is made into the cerebrum.<sup>16</sup> Would infection therefore be prevented if an immune serum was injected into the meninges previous to the intravenous administration of the virus? For if infection does not occur, the difference would be attributable only to the presence within the immune serum of the neutralizing substances for the virus.

*Experiment 18.*—Dec. 21, 1916. 5 p.m. 2 cc. of immune monkey serum pooled from three animals which had recovered from an attack of experimental poliomyelitis were injected intraspinally into a *Macacus rhesus*. Dec. 22, 11 a.m. 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla of a paralyzed monkey were injected intravenously. No symptoms appeared.

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<sup>15</sup> Flexner, S., *J. Am. Med. Assn.*, 1910, lv, 1105.

<sup>16</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1780; lv, 662.

This experiment is conclusive in that it indicates that an immune serum alone of all the irritative fluids injected into the meninges prevents the infection. The experiment has a bearing also on the serum therapy of human poliomyelitis and upon the question of the employment for intraspinal injection of normal sera and other fluids of more or less irritative character.

The next series of experiments relates to the protecting power of an immune serum when it is employed to overcome the effect on the infection of an intraspinal injection, either of normal monkey serum or of normal salt solution. That protection would thus be afforded by the immune serum was regarded as certain, in view of the power which it had already displayed to hold up an infection by means of the intravenous administration of the centrifuged virus following the intraspinal injection of horse serum.<sup>8</sup>

*Experiment 19.*—Dec. 19, 1916. 4.30 p.m. 2 cc. of normal monkey serum were injected intraspinally into a *Macacus rhesus*. Dec. 20, 1 p.m. 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla were injected intravenously. 1.30 p.m. Intraspinally injection of 3 cc. of pooled immune monkey serum. Dec. 26. Ataxia; head tremor; ptosis. Dec. 27. Symptoms more marked. Dec. 28. Widespread weakness of muscles. Dec. 30. Prostrate. This animal slowly recovered with marked residual paralysis.

*Experiment 20.*—Dec. 19, 1916. 2 cc. of normal monkey serum were injected intraspinally into a *Macacus rhesus*. Dec. 20, 12 noon. 50 cc. of a centrifuged 5 per cent suspension similar to that used in the previous experiment were injected intravenously. 12.20 p.m. 3 cc. of pooled immune serum were injected intraspinally. The immune serum was given on the next 3 successive days, and after a 2 days' rest on 3 more successive days. No symptoms developed.

*Experiment 21.*—This experiment was an exact repetition of Experiment 20, and yielded the same results.

The inflammatory changes induced by normal monkey serum similar to those brought about by normal horse serum require, apparently, more than a single injection of the immune serum to prevent infection after intravenous injection. Two or three injections at 24 hour intervals would suffice, for within that period the defect would have probably been healed and the major part of the virus neutralized. Moreover, it has been shown<sup>17</sup> that large doses of the virus injected into the blood begin to disappear in 96 hours and the virus

<sup>17</sup> Flexner and Amoss, *J. Exp. Med.*, 1914, xix, 411.

no longer circulates in detectable quantities after 120 hours. After inflammation has been produced in the meninges by normal monkey serum a single intraspinal injection of the immune serum mitigated the severity of the infection, since, contrary to the rule, the paralyzed animal lived.

The last experiment of this series was made with normal salt solution. It repeats exactly the one in which normal monkey serum was injected intraspinally, the centrifuged virus intravenously, and one dose of immune serum intraspinally. In conformity with the smaller damage inflicted upon the meningeal structures, a single dose of the immune serum is observed to prevent infection.

*Experiment 22.*—Jan. 15, 1917. 3.30 p.m. 2 cc. of normal salt solution were injected intraspinally into a *Macacus rhesus*. Jan. 16, 10 a.m. 50 cc. of a centrifuged 5 per cent suspension of active spinal cord and medulla were injected intravenously. 10.20 a.m. Intraspinal injection of 3 cc. of pooled immune serum. No symptoms developed.

This group of experiments confirms and extends somewhat the earlier ones made with normal horse serum. It now appears not only that an immune serum injected intraspinally is protective, but that the degree of its efficiency is more or less proportional to the injury which the meningeal structures have suffered from the irritating substances employed to increase their permeability. The slighter the inflammation, the more readily and quickly the injury is repaired; with isotonic salt solution a single dose of the immune serum suffices to prevent infection, while with monkey and horse serum several doses are required.

#### CONCLUSIONS.

Among the mechanisms which defend the body from infection with the virus of poliomyelitis is the meningeal-choroid plexus complex, which normally is capable of excluding the circulating virus from the central nervous organs. The complex plays a part also in preventing infection from virus present upon the nasal mucosa.

Aseptic fluids which irritate, inflame, or even slightly alter the integrity of the meninges and choroid plexus diminish or remove their protective function.

Normal monkey or horse serum, isotonic salt solution, and Ringer's



and Locke's solutions, when injected into the meninges, promote infection with the virus of poliomyelitis introduced into the blood, the nose, or the subcutaneous tissues.

Simple lumbar puncture and the withdrawal and return of the cerebrospinal fluid in normal monkeys, hemorrhage having been absolutely avoided, do not promote infection with virus injected into the blood; while the replacement of the cerebrospinal fluid of one monkey with that of another does in some instances lead to infection. Simple lumbar puncture attended with even very slight hemorrhage opens the way for the passage of the virus from the blood into the central nervous tissues, and thus promotes infection.

Hence, changes in the structure or function of the meningeal-choroid plexus complex, too slight to be detected by chemical and cellular changes in the cerebrospinal fluid or by morphological alterations, suffice to diminish in an essential manner its protective powers.

Of all the irritant fluids tested, immune serum alone injected into the meninges is not succeeded by infection from the virus introduced into the blood.

The protective property of the immune serum is capable of overcoming the promoting action of normal monkey and horse serum and the other irritants mentioned.

The importance first of the meningeal-choroid plexus complex in preventing infection with the virus of poliomyelitis, and next of immune serum in offsetting the disadvantages and dangers arising from defects in the mechanism is apparent, as is the bearing of the experiments reported on the serum therapy of epidemic poliomyelitis.





## SURVIVAL OF THE POLIOMYELITIC VIRUS FOR SIX YEARS IN GLYCEROL.

BY SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 40 TO 43.

(Received for publication, February 6, 1917.)

The reappearance of poliomyelitis in severe form in the United States during the summer and autumn of 1916 has revived the question of the conditions under which the microbic cause, or virus, of the disease survives outside the human body. The problem of the source of the epidemic is still unsolved: whether, for example, the virus was newly imported or merely a survival from previous epidemics of the disease. We are not now in a position to answer this question. Our present knowledge indicates that the virus does not multiply in nature outside the human body, but it is possible that it may survive upon human carriers or elsewhere without actually multiplying.

The question of the power of the virus to resist extraneous influences has been sharpened by recent publications connecting the streptococcus with the etiology of poliomyelitis. Experience has shown that streptococci in animal tissues, for example, do not retain viability over long periods of time. We ascertained early in the experimental investigation of poliomyelitis that the virus in the central nervous tissues resists glycerolation,<sup>1</sup> and later we reported instances of survival after 25 months' immersion in glycerol.<sup>2</sup> In the course of investigations on poliomyelitis conducted at The Rockefeller Institute, specimens of nervous tissues from human beings and monkeys, the subjects of poliomyelitis, have been regularly put aside in 50 per cent glycerol in the refrigerator, the temperature of which has been

<sup>1</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 45.

<sup>2</sup> Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 205.

kept at approximately 4°C. Some of the tissues were set aside in 1910, and others later. We have recently tested certain specimens for activity and ascertained that monkeys may be infected with samples 6 and 4 years old.

The spinal cord and medulla were cut into small cubes which were immersed in a large excess of 50 per cent glycerol previously sterilized in the autoclave. When portions were removed for purposes of inoculation, tests were made for bacteria by imbedding fragments of the tissue in deep tubes of glucose-ascitic-fluid-agar and inoculating the tissue on the surface of tubes of that medium. The tubes were incubated for several days. On microscopic examination no growth was discovered.

*Experiment 1.*—Human spinal cord and medulla kept in glycerol since August, 1911, and September, 1912. Fragments from the two specimens were combined for inoculation. Hence the more recent sample of tissue had been put aside more than 4 years before. Dec. 7, 1916. A *Macacus rhesus* was given an intracerebral injection, under ether anesthesia, of a heavy suspension of the tissues. Dec. 11. 3 cc. injected into the peritoneal cavity. Jan. 4, 1917. No symptoms. A fresh suspension was prepared of the same tissues, of which 3 cc. were given intracerebrally and 5 cc. intraperitoneally. Jan. 17. Excitement; ataxia; tremor of head; facial asymmetry; weakness of right deltoid. Jan. 22. The symptoms have advanced slightly. Jan. 27. Injected 2 cc. of suspension intracerebrally and 5 cc. intraperitoneally. Jan. 31. Symptoms slowly increasing. Etherized for histological examination and for purposes of reinoculation.

*Histology.*—Lesions occur in the medulla, spinal cord, and intervertebral ganglia. Those present in the medulla consist (1) of lymphocytic infiltration of the vessels in the meninges and (2) of slight perivascular infiltration of the superficial internal vessels. There is no general lymphocytic infiltration and no necrosis of nerve cells. The lesions in the spinal cord are focal. The cervical cord shows diffuse lymphocytic infiltration, necrosis of anterior horn nerve cells, and a small degree of neurophagocytosis. The blood vessels of the gray matter are moderately infiltrated with mononuclear cells. The lumbar cord is the seat of marked perivascular infiltration affecting chiefly the gray matter, but the meningeal vessels are also heavily surrounded (Fig. 1). The anterior gray matter is diffusely, but not severely, infiltrated with lymphocytic cells and no neurophagocytosis is present in the sections studied. The intervertebral ganglia show slight infiltration with lymphocytes and no nerve cell degeneration.

*Experiment 2.*—Monkey spinal cord and medulla. Several specimens of tissue from monkeys inoculated with M. A. virus in the spring and autumn of 1910 were combined for testing. They had been kept in 50 per cent glycerol for 6 years or longer at 4°C. Culture tests, similar to those described in Experi-

ment 1, were negative. Dec. 9, 1916. A *Macacus rhesus* received, under ether anesthesia, an intracerebral inoculation of 3 cc. of a heavy suspension. Dec. 12. 13 cc. of the same suspension were injected into the peritoneal cavity. Jan. 4, 1917. No symptoms had appeared. Injected 2 cc. of a fresh suspension made from the same tissues intracerebrally and 7 cc. intraperitoneally. Jan. 13. Ataxia; shoulder and leg muscles weak. Jan. 14. Weakness advancing; animal unable to climb. Jan. 17. Prostrate. Etherized.

*Histology.*—Sections prepared from the medulla, spinal cord, and intervertebral ganglia were studied. The medulla shows marked perivascular lymphocytic infiltrative lesions of the meninges and less of the nervous tissue itself. But within the latter are small focal lymphocytic accumulations sometimes about degenerating nerve cells. The spinal cord shows more pronounced lesions, especially of the anterior gray matter. Diffuse lymphocytic and edematous infiltration as well as marked perivascular cellular infiltrations are prominent (Fig. 2). The blood vessels of the white matter are widely affected. Hyaline degeneration of motor nerve cells is frequent, but little neurophagocytosis is going on (Fig. 3). The intervertebral ganglia show typical focal infiltrative lymphocytic lesions, but no necrosis of ganglion cells (Fig. 4).

The lesions in the nervous organs of the second monkey exceed in extent and severity those of the first. This is in conformity with the greater pathogenicity displayed for monkeys of the virus which has become adapted to that species.

The experiments described show that the virus of epidemic poliomyelitis as contained in the central nervous organs of human beings, the subjects of epidemic poliomyelitis, and of monkeys in which the experimental disease has been induced, survives for many years in the weak disinfectant glycerol. The manner in which thereinoculations which led to paralysis and to characteristic histological lesions were made indicates that during the periods mentioned, the virus suffered reduction in activity either because of mere diminution in the number of microorganisms still surviving or through qualitative modifications of the viable organisms themselves.

On the other hand, the experiments again bring out the value of reinoculation in establishing the viability and activity of the microorganism causing poliomyelitis.<sup>3</sup> The virus of this epidemic disease is peculiar in that the injection into the monkey does not afford an immunity unless obvious symptoms of an infection appear. An unsuccessful inoculation increases susceptibility to a subsequent

<sup>3</sup> Flexner, S., Noguchi, H., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 91.

injection of the same relatively ineffective material. It seems as if an accumulation of the virus takes place until the quantity or number of viable microorganisms becomes sufficient to induce an infection. Repeated inoculations, therefore, rather lead to infection than to specific protection. This phenomenon was noted first with the active monkey virus used in subminimal doses for purposes of immunization<sup>4</sup> and later with the globoid bodies<sup>5</sup> of which a single inoculation failed, but repeated injections sufficed to cause experimental poliomyelitis.<sup>3</sup>

The demonstration of the survival of the virus in the presence of a weak antiseptic compound for the long periods given may have a bearing on the epidemiology of poliomyelitis. The reappearance in a given place of epidemic disease is usually ascribed to a new importation of virus, when possibly the explanation is sometimes to be sought in the persistence and survival of specimens of the virus which flourished previously in that or a neighboring community.

#### CONCLUSIONS.

The virus of poliomyelitis contained within the spinal cord and medulla of human beings and monkeys withstands glycerolation for many years. The specimens tested had been kept for 4 and 6 years respectively in 50 per cent glycerol at refrigerator temperature.

The symptoms and lesions caused by this virus are identical with those produced by that contained in the more recently collected spinal cord and medulla.

The specimens had lost a part of their activity under the conditions described, necessitating larger and repeated doses to induce infection. Whether this difference is due merely to quantitative reduction in number of viable microorganisms or to qualitative alterations under the influence of the mildly antiseptic glycerol has not been determined.

An ineffective inoculation of tissues containing the virus does not increase resistance, but rather diminishes it, so that a subsequent injection, inadequate in itself, may cause experimental poliomyelitis.

This power of survival under adverse conditions may not be without

<sup>4</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1780.

<sup>5</sup> Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

significance in respect to the recrudescence of poliomyelitis in a given locality and after a lapse of years. Hitherto this phenomenon has been accounted for by assuming a fresh importation of a virus of pronounced pathogenic power. It is possible that the explanation in some instances resides in the renewed activity of specimens of the virus surviving from a previous epidemic, while in other instances a fresh introduction actually takes place from a remote focus of infection.

The infectious nervous tissues employed in these experiments did not yield in culture streptococci or other ordinary bacteria.

#### EXPLANATION OF PLATES.

##### PLATE 40.

FIG. 1. Spinal cord of the monkey in Experiment 1, showing perivascular infiltration of meningeal and intramedullary vessels and focal accumulations of lymphoid cells.  $\times 62$ .

##### PLATE 41.

FIG. 2. Spinal cord of the monkey in Experiment 2, showing perivascular and diffuse lymphocytic infiltration of the white and gray matter.  $\times 62$ .

##### PLATE 42.

FIG. 3. The same as Fig. 2. Perivascular infiltrative necrosis of nerve cells and neurophagocytosis.  $\times 400$ .

##### PLATE 43.

FIG. 4. Intervertebral ganglion of the monkey in Experiment 2, showing focal accumulations of lymphoid cells.  $\times 62$ .







FIG. 1.

(Flexner and Amoss: Survival of Poliomyelitic Virus.)



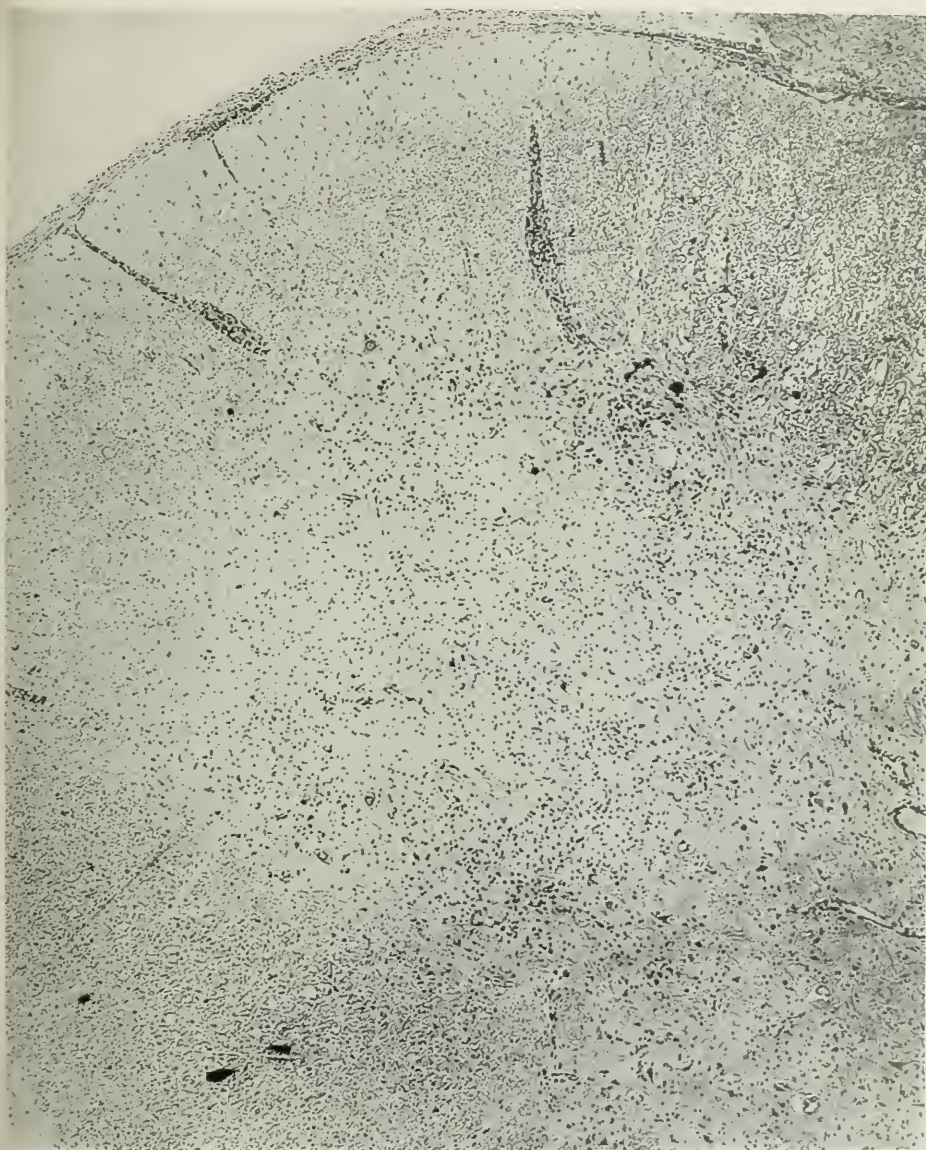


FIG. 2.

(Flexner and Amoss: Survival of Poliomyelitic Virus.)



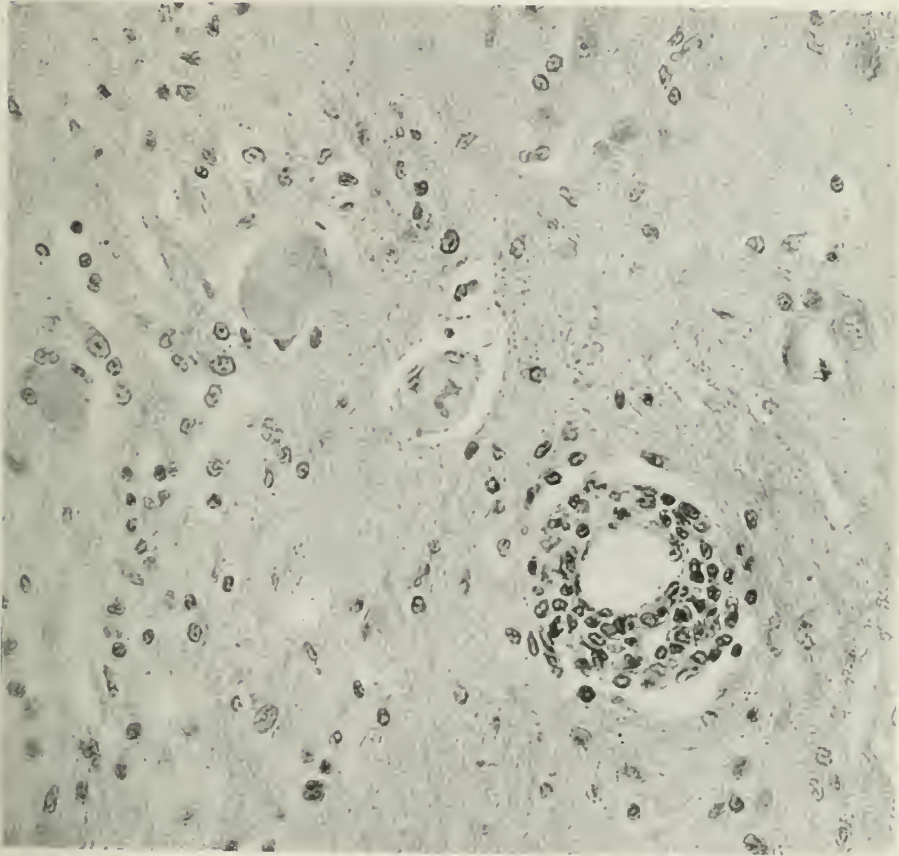


FIG. 3.

(Flexner and Amoss: Survival of Poliomyelitic Virus.)





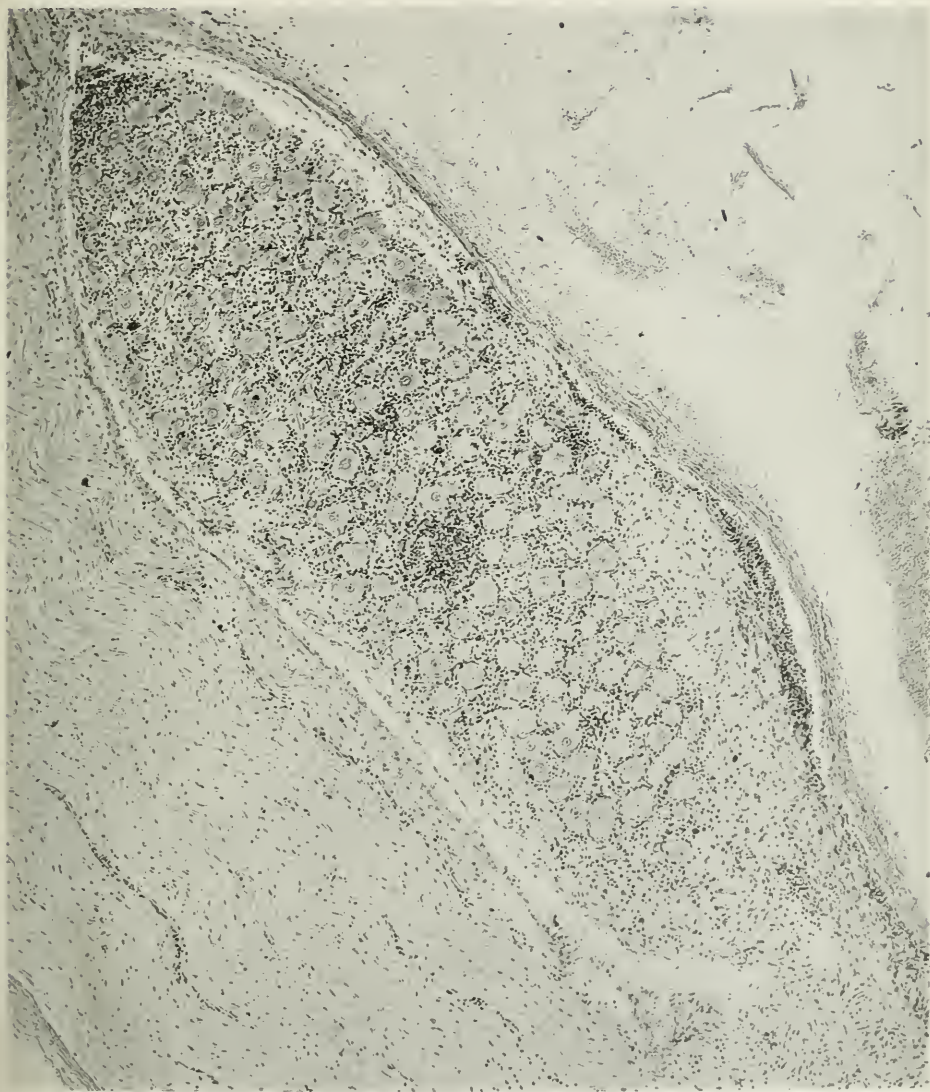


FIG. 4.

(Flexner and Amoss: Survival of Poliomyelitic Virus.)



# THE CULTIVATION AND IMMUNOLOGICAL REACTIONS OF THE GLOBOID BODIES IN POLIOMYELITIS.

By HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 44 TO 46.

(Received for publication, December 5, 1916.)

The microorganism described by Flexner and Noguchi<sup>1</sup> under the name of globoid bodies has been shown to bear a definite relation to epidemic poliomyelitis. The bodies are obtained in cultures with great difficulty and thus far only those who have employed the original method of cultivation have been rewarded with successful results. This is significant in respect to the etiology of poliomyelitis, for, while a variety of ordinary bacteria easily cultivatable has been obtained by various workers, the globoid bodies represent a peculiar species and thus far alone have sufficed to produce typical poliomyelitis in monkeys by intracerebral inoculation.

The globoid bodies may be said in general to have fulfilled Koch's law of causation. They have been found repeatedly in the lesions of poliomyelitis in man and the monkey; they have not been detected in lesions or conditions other than poliomyelitis; they have sufficed to reproduce in several instances the experimental disease in monkeys; and they have been recovered in cultures from the lesions thus produced.

But the globoid bodies have often not been obtained in cultures, even with Noguchi's technique.<sup>2</sup> At first sight this fact seems to cast doubt on the nature of the globoid bodies and to give rise to the question whether they are not occasional and hence not necessary accompaniments of the lesions of poliomyelitis, or, in other words, whether they constitute the microbic cause of poliomyelitis. The doubts raised by this question can at the outset be met by pointing

<sup>1</sup> Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

<sup>2</sup> Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

to the successful inoculations, although they are not numerous. The monkeys so inoculated developed typical poliomyelitis both symptomatically and histologically. Moreover, the nervous tissues of these animals, like the filtered virus, reproduced poliomyelitis in monkeys. Filtrates of the spinal cord and medulla and of the lesion at the local site of injection of the cultivated globoid bodies were characteristically actively infectious. Moreover, the cultures in different generations set up typical experimental poliomyelitis, and as long as 20 months after isolation and not only after a single inoculation, when most active, but subsequently, after repeated inoculation, when the activity was somewhat diminished.<sup>3</sup>

The globoid bodies, moreover, have been detected directly under the microscope in the nervous tissues of human beings and monkeys, the subjects of epidemic poliomyelitis,<sup>1</sup> and in one instance in films prepared from the circulating blood of a monkey experimentally infected with the filtered virus,<sup>4</sup> and in conformity with this observation they have been cultivated in one instance from the circulating blood of a monkey injected with a virus filtrate.

To one familiar with the properties of the filtered virus, this is significant. The virus filtrate does not withstand well thermostat temperatures and the presence of colloidal substances possessing absorptive power.<sup>5</sup> Under these conditions its activity is totally lost in from a few hours to a day or two. An injection of relatively inactive filtrate virus in monkeys does not produce resistance, but either leaves the animal unchanged immunologically or induces a state of heightened susceptibility to which, upon subsequent injection of the same virus, days or weeks later, the animal responds with typical poliomyelitic paralysis. The globoid bodies have been shown to be capable of behaving in precisely the same way.<sup>3</sup>

It should be recalled that the technique employed in the cultivation of the globoid bodies is based upon Noguchi's method of cultivating *Treponema pallidum*.<sup>2</sup> The conditions surrounding the cultivation of the two microorganisms directly from infected tissues are strikingly similar. Experience with the one is almost invaluable

<sup>3</sup> Flexner, S., Noguchi, H., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 91.

<sup>4</sup> Amoss, H. L., *J. Exp. Med.*, 1914, xix, 212.

<sup>5</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 509.

for success with the other. The presence of the *pallidum* in large numbers in the testicular lesions of rabbits is readily demonstrated under the dark-field microscope and by stained preparations. But bacteriologists who have attempted for the first time to cultivate the *pallidum* directly from the testicle have usually met with many difficulties and not infrequently have failed entirely.

Microorganisms as highly parasitized as the *pallidum* and the globoid bodies appear to require special conditions for growth in artificial cultures. As yet the latter have not been secured directly in a solid medium. In sharp contradistinction to this is the relative ease with which subculture succeeds, although not in every instance. Certain cultures both of the *pallidum* and the globoid bodies do not lend themselves to subculture, probably because the necessary conditions of multiplication have not been maintained in the artificial media.

Once cultivation of the strains is established, the organisms become, in time, more and more saprophytized and, as will be described in this paper, relinquish requirements, such as the presence of fresh animal tissue, indispensable at the outset. When this state arises, pathogenicity has, as a rule, been completely lost. On the other hand, as the medium has now become less complex, the conditions for the carrying out of immunological reactions have become more favorable. In one respect no change has been noted in the nature of the globoid bodies: they have remained anaerobic.

### *Cultivation.*

When a strain of the globoid bodies has been accustomed to artificial cultivation, which usually requires several transplantations of the first cultures, growth is more readily and abundantly obtained. Apparently an adaptation to the new conditions gradually takes place. It is at first impossible to obtain large cultures, mass cultures so called; however, after several generations they may be grown in Erlenmeyer or Florence flasks of suitable size, in a medium consisting of ascitic fluid and agar containing a fragment of rabbit kidney. After congelation the substratum is overlaid with bouillon which in turn is covered with a layer of sterilized paraffin oil.<sup>3</sup> The growth



first takes place throughout the substratum and then extends into the bouillon, which, however, does not become turbid. The organisms appear not to multiply in the broth, but give rise to a sediment overlying the agar base. Later a mass may be obtained by collecting this sediment from several flasks. Since the bouillon will not support the growth of globoid bodies, degenerations set in quickly, the organisms showing striking irregularities in size and staining property.

The tests were carried out with four strains of the globoid bodies obtained as follows: Two cultures were original Noguchi strains obtained in 1914 from specimens of M. A. virus<sup>1</sup> which had been passed through many monkeys; the other two strains were obtained by the author in 1915, one from the M. A. virus, and the other from monkeys inoculated with a sample of human virus. The four cultures were indistinguishable morphologically and biologically. When they were subjected to the modification of culture media to be described, two strains had been under artificial cultivation for 2 years and the other two for 1 year or longer.

*Kidney.*—The indispensable animal tissue employed in the original cultivations has been fresh rabbit kidney. Noguchi believes that the tissue performs the double function of withdrawing the last traces of oxygen from the medium and of providing an essential nutritious element. Growth first appears about the tissue fragment and then extends peripherally. The tests made relate (a) to boiled kidney and (b) to dispensing with the fragment of organ altogether.

(a) The kidney fragments are dropped into boiling sterile distilled water and kept there until coagulation is completed. They are employed in the manner of fresh fragments in the semisolid ascitic agar medium. All the cultures multiplied, but the growth was less luxuriant than with the fresh organ.

(b) Growth has also taken place in complete absence of the tissue fragment, but it is even less abundant than in the presence of the coagulated tissue.

The saprophytized cultures have, therefore, become essentially altered in their extreme oxygen and food requirements. They appear to be less sensitive to oxygen and less exacting nutritionally.

*Broth and Ascitic Fluid.*—None of the cultures multiply in broth, even in the presence of the kidney fragment, and while some growth

is obtained in pure ascitic fluid, it is feeble. When the broth and ascitic fluid are mixed in equal proportion, growth is more abundant. The addition of 1 per cent dextrose does not improve the growth and no acid is formed. Ascitic fluid inactivated at 56°C. is unsuitable for the original cultivation of the globoid bodies, but the adapted cultures flourish equally well in the inactivated fluid.

*Blood Serum.*—Blood serum is not suitable for obtaining original isolations. In the presence of the kidney fragments the adapted cultures multiply freely in monkey serum, less in horse and rabbit serum, and least in human serum. The omission of the kidney has the effect of diminishing materially the amount of growth and wiping out the qualitative distinctions of the several sera. A 1:5 dilution of the sera with bouillon neither favors nor hinders this growth.

The saprophytized cultures have, therefore, undergone fundamental changes in growth requirement. The changes have produced insignificant alterations in morphology. On the whole, the saprophytized globoid bodies are somewhat less tenuous, perhaps a trifle larger, and stain more deeply with Giemsa and other stains. Their reaction to Gram's stain is also somewhat irregular, depending upon the medium employed, but they remain essentially Gram-negative. Combined artificially with cultures of streptococci and stained either with Giemsa's or Gram's stains, the distinction is striking (Fig. 1). None of the saprophytized cultures displayed pathogenic power for monkeys. Large quantities were injected intravenously into rabbits without producing any detectable ill effect. Since the cultures could now be obtained free of human protein, they were better adapted to immunological studies.

### *Immunology.*

Our knowledge of immunological reactions in poliomyelitis is small. The one biological test for determining the presence and perhaps the amount of immune substances is that of neutralization.<sup>6</sup> From the fact that the blood serum of recovered human beings is protective and probably curative in poliomyelitis, it is also assumed that immune bodies are present in the fluid.

<sup>6</sup> Landsteiner and Levaditi, *Compt. rend. Soc. biol.*, 1910, lxviii, 311.

The particular object of this study of the immunological reactions of the globoid bodies was additional information on their relation to poliomyelitis in man and the monkey infected with the filterable virus.

This investigation was begun with considerable doubt of obtaining a clear-cut result. The difficulties surrounding the usual immunological tests with *Treponema pallidum*, with the cultural conditions of which the globoid bodies exhibited so many analogies, were before us.<sup>7</sup> Rabbits and monkeys were injected with the globoid bodies in order to develop antisera which might show agglutination and complement deviation, and the sera of human beings and monkeys which had recovered from attacks of poliomyelitis were tested against cultures of the globoid bodies. In order to increase their resistance, the monkeys, after recovery, had received subcutaneous injections of the emulsified active spinal cord and medulla of paralyzed animals which had succumbed.

*Rabbits.*—Two series of rabbits of three each received repeated intravenous inoculations of mass cultures of four strains. The first series received from 0.5 to 5 cc. of a heavy suspension of washed cultures of globoid bodies, and the second received injections from 0.5 to 5 cc. on 3 successive days, and were allowed to rest 7 days, according to the method of Flexner and Amoss for the rapid production of antidysenteric and antimeningococcic sera.<sup>8</sup>

With the exception of two animals that died of snuffles, the rabbits showed no reaction or symptoms, and at the end of 3 months they were well and had not lost weight. During and at the end of this time, the serum exhibited no differences in immunological reactions when compared with the normal except that in two rabbits there was slight fixation, and in one 0.1 cc. completely fixed 0.04 cc. of guinea pig complement in the presence of 0.2 cc. of antigen prepared from mass cultures. 2 cc. of the serum which exhibited the power of complement fixation, however, failed to neutralize 0.1 cc. of the filterable virus.

It is noteworthy that no clinical or immunological effect was produced in rabbits. They did not become sensitized to the globoid

<sup>7</sup> Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1916, xxiv, 561.

<sup>8</sup> Flexner and Amoss, *J. Exp. Med.*, 1915, xxi, 515. Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

bodies, nor did they respond, with one exception, with the development of detectable antibodies.

*Monkeys.*—Eleven monkeys received regular weekly injections of mass cultures of the four strains of the globoid bodies over a period of 2 years, and their serum was tested at intervals during that time. Of the eleven monkeys two received 5 cc. of a heavy suspension of the mass cultures (one recently, the other remotely isolated) intraspinally each week for 1 year, and intravenous injections of the same amounts weekly for another year. Three received 12 intraspinal injections and 28 intravenous injections; two received 5 injections intraspinally, and 35 intraperitoneally; two received a single injection intracerebrally and multiple injections intravenously; and two received intraperitoneal and intravenous injections.

Only the monkeys receiving the cultures subdurally exhibited symptoms suggestive of poliomyelitis which never progressed, however, beyond the stage of excitability, ruffling of the fur, and staccato cries.

The serum of only two monkeys, those receiving repeated intraspinal injections, developed the power of fixing complement. All the sera to a greater or less degree inhibited the growth of the globoid bodies and produced the thread reaction described below.

As has been indicated, the monkeys that received cultures of the globoid bodies over a considerable period of time were not wholly indifferent to the injections. They were refractory like the rabbits, but in two instances a reaction with antibody production was apparent. The reaction may be considered as affecting agglutination and complement deviation.

Since the globoid bodies grow in loose masses in fluid media, the ordinary methods of testing for agglutination were not satisfactory. The employment of normal and immune monkey serum in the medium of cultivation yielded sharper effects. Cultures were made in a medium composed of 1 part of the serum to be tested plus 4 parts of neutral broth.

In the presence of kidney tissue no difference can be detected between the rate and amount of growth in normal serum and immune serum. However, when kidney tissue is omitted, marked differences are apparent. Serum from monkeys immunized by intraspinal or



intravenous injection of the organism over long periods produced the most marked effect in retarding the growth of the globoid bodies. The serum from monkeys which had recovered from experimental poliomyelitis induced by the filtrate virus and which were afterwards reinforced inhibited the growth, but in less degree (Fig. 2).

### *Morphology.*

The film preparations were made in a uniform manner. They were air-dried, fixed in methyl alcohol for 1 minute, and stained over night in 1:10 Giemsa solution and by Gram's method.

The control cultures in the ascitic-fluid-broth-kidney medium yielded cultures growing in bunches with no special arrangement of individuals. When no kidney tissue is present the chain formation is more prominent and the staining is faint. The controls in normal monkey serum with broth also give large bunches of faintly staining organisms (Fig. 3).

On the other hand, the serum of monkeys injected with the active cultures produces a marked change in the size and arrangement of the globoid bodies. The chain production is intensified and they interlace, so as to resemble the Pfaundler reaction of typhoid bacillus agglutination (Fig. 4). The length of many of the chains may be inferred from one chain in which 230 individuals were counted. The individual bodies also become larger and may become elongated. The maximum alteration is produced in the first generation in the immune serum; after several transfers in immune serum a single generation in a non-immune medium suffices to restore the original grouping and size to the cultures.

The serum from recovered and reinforced monkeys retards the growth of the globoid bodies, but less than that of the monkeys treated with the cultures. The chain formation is less also and the enlargement of the individual bodies, while apparent, is also less conspicuous (Fig. 5).

### *Complement Deviation.*

For this test the serum (a) of monkeys treated with the cultures of the globoid bodies, (b) of recovered and reinforced monkeys, and (c) from paralyzed cases of poliomyelitis in man was employed.

*Antigen.*—For the preparation of the antigen, mass cultures were used in the manner described above, in small Florence flasks, as recommended by Zinsser.<sup>9</sup> The mass was washed in sterile water and suspended again in sterile water. It was alternately frozen and thawed three times, heated to 56°C. for 2 hours, transferred to a mortar, and the water evaporated at 45°C. under a current of filtered air. 1 cc. of water was added and the heavy suspension ground with glass. 20 cc. of water were added and the suspension was shaken for 30 minutes. After centrifuging at high speed, the fluid was passed through a Berkefeld filter, and made isotonic by the addition of 0.9 per cent sodium chloride solution. Extracts prepared in this manner were not anticomplementary, but rapidly lost antigenic power at 4°C.

Rabbit anti-hen hemolytic system was used when monkey serum was titrated, and rabbit anti-sheep hemolytic system when human serum was tested. Monkey serum becomes slightly anticomplementary when inactivated by heat, but the removal of complement by allowing the serum to remain at 4°C. for 4 days produces no anti-complementary action.

Serum from ten monkeys which had been injected with the cultures of globoid bodies was employed. In only two instances were reactions obtained. The two positive results were with monkeys which had received eighteen intraspinal injections of a mass culture of the globoid bodies, and later twenty-one weekly intraperitoneal injections of similar material. The serum of these animals fixed two units, or 0.4 cc., of guinea pig complement in the presence of 0.1 cc. of antigen.

The sera were subsequently tested upon the active filtrate virus and failed to show neutralizing power.

The antigen which reacted to the two sera of monkeys injected with the cultures yielded no complement fixation with the sera of monkeys which had recovered from experimental poliomyelitis or with the blood taken from twenty-six human beings, chiefly children, suffering from epidemic poliomyelitis. The latter specimens were taken for the most part in the early stages of an acute attack.

Incidentally, it may be stated that antigens were prepared from human poliomyelitic glands and from the spleen, bone marrow, lymph glands, and nasal mucosa of monkeys succumbing to experimental

<sup>9</sup> Zinsser, H., Hopkins, J. G., and Gilbert, R., *J. Exp. Med.*, 1915, xxi, 213.



poliomyelitis, and tested against the human blood sera. The result in every instance was negative.

Finally, the saprophytized cultures of the globoid bodies were cultivated through several generations in serum from monkeys injected with globoid bodies, from monkeys recovered from experimental poliomyelitis, and from human cases of poliomyelitis. The cultures so obtained were injected intracerebrally, under ether anesthesia, into *rhesus* monkeys but in no instance was paralysis induced.

At the outset we drew attention to the similarities in conditions of culture displayed by the globoid bodies and *Treponema pallidum*; here we desire to emphasize the analogies in immunological reaction exhibited by the culture and parasitic types of the two microorganisms respectively.<sup>7</sup>

#### CONCLUSIONS.

Two additional cultures of globoid bodies, obtained from the nervous tissues of monkeys in which experimental poliomyelitis was produced, and identical with the original cultures described by Flexner and Noguchi, are reported in this paper.

The highly parasitic cultures, like *Treponema pallidum*, are refractory to artificial cultivation.

After long cultivation outside the body, the globoid bodies acquire saprophytic properties and then grow more readily and in a considerable variety of media, provided, however, that they carry a certain quantity of protein matter not denatured.

The rabbit responds slightly with the production of antibodies to the injection of cultures of the globoid bodies.

The monkey responds only occasionally under the same conditions and apparently only when the cultures are injected into the central nervous system.

This response is small and at most leads to slight reactions of agglutination and complement deviation with the cultures.

The serum obtained from monkeys recovered from experimental poliomyelitis shows even less agglutination and no complement deviation.

The maximal agglutinative and morphological changes produced in the globoid bodies cultivated in immune monkey serum are ob-

tained in the first generation; after several subcultures in the immune serum, the reversion to normal takes place in one generation in a non-immune medium.

The serum of human beings in the acute or early subacute stages of poliomyelitis gives no complement deviation with the antigen derived from the globoid bodies.

The cultivation of the saprophytized globoid bodies through several generations in immune monkey or human serum did not confer upon them pathogenic properties for monkeys.

The serum of human beings and monkeys which have survived attacks of poliomyelitis does not fix complement in the presence of antigens prepared from organs of the monkey succumbing to the experimental disease.

The globoid bodies and *Treponema pallidum* present many analogies in cultural, immunological, and pathogenic properties.

#### EXPLANATION OF PLATES.

##### PLATE 44.

FIG. 1. Film preparation of globoid bodies (violet); *Streptococcus pyogenes* (blue). Giemsa 1: 10, 16 hours at room temperature.  $\times 1,000$ .

##### PLATE 45.

FIG. 2. A. Culture of globoid bodies in a medium composed of 4 parts of bouillon and 1 part of normal monkey serum. No kidney tissue.

B. Culture of globoid bodies in a medium composed of 4 parts of bouillon and 1 part of serum, from a monkey which had received repeated injections of mass cultures of globoid bodies. No kidney tissue.

C. Culture of globoid bodies in a medium composed of 4 parts of bouillon and 1 part of serum, from a monkey which had recovered from experimental infection with the filterable virus. No kidney tissue.

##### PLATE 46.

FIG. 3. Film preparation from Tube A (Fig. 2). Giemsa stain 1: 10, 16 hours at room temperature.  $\times 1,000$ .

FIG. 4. Film preparation from Tube B (Fig. 2). Giemsa stain 1: 10, 16 hours at room temperature.  $\times 1,000$ .

FIG. 5. Film preparation from Tube C (Fig. 2). Giemsa stain 1: 10, 16 hours at room temperature.  $\times 1,000$ .



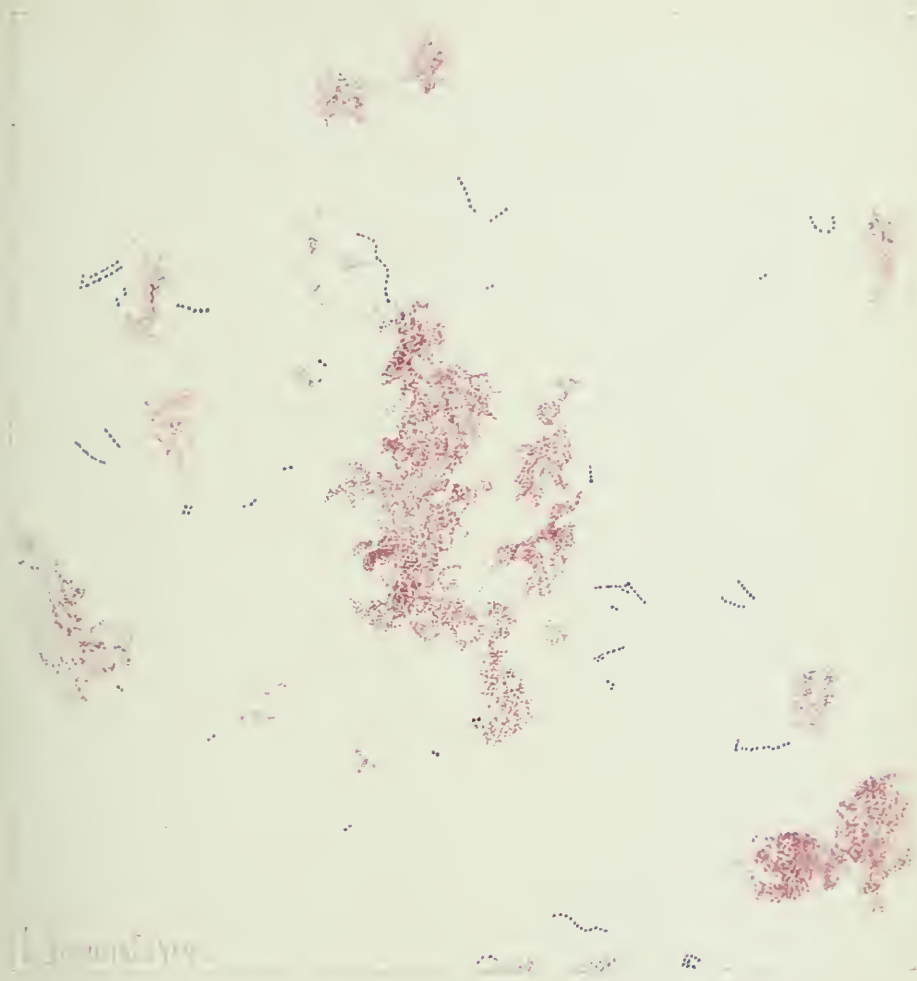


FIG. 1.

(Amoss: Globoid Bodies in Poliomyelitis.)





FIG. 2.

(Amoss: Globoid Bodies in Poliomyelitis.)





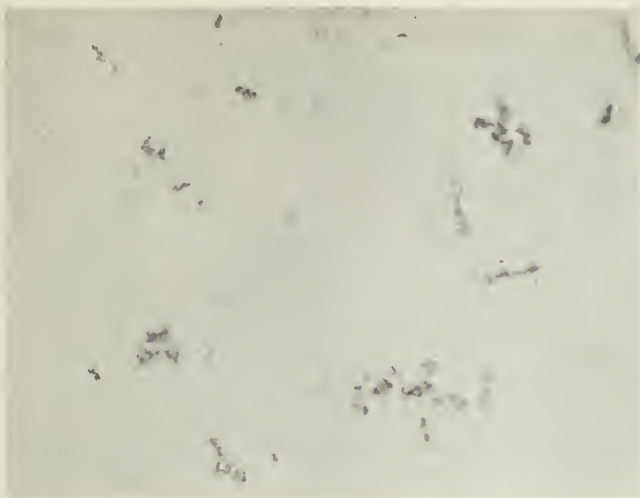


FIG. 3.

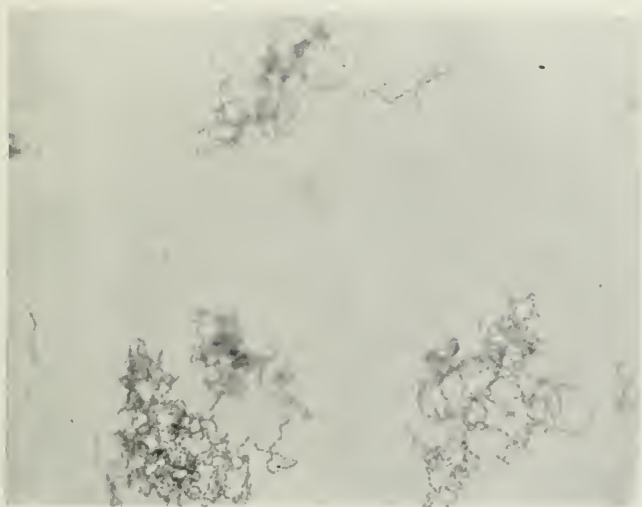


FIG. 4.

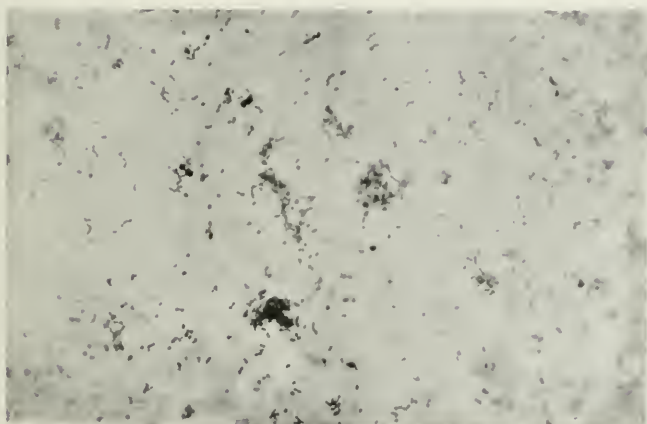


FIG. 5.

(Amoss: Globoid Bodies in Poliomyelitis.)



# THE PATHOLOGIC EFFECTS OF STREPTOCOCCI FROM CASES OF POLIOMYELITIS AND OTHER SOURCES.

By CARROLL G. BULL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 47 TO 53.

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The etiology of epidemic poliomyelitis has again become one of the debated questions in pathology. While it is not denied that the filterable virus<sup>1</sup> is the inciting cause of the disease in man and the monkey, the particular question which has now arisen is the relation of the streptococcus to the filterable organism.

Several authors<sup>2</sup> have recently reported almost simultaneously on the streptococcus as capable of setting up, on inoculation into animals, clinical and pathologic states which they identify with poliomyelitis in man and the experimental poliomyelitis induced in monkeys by inoculation of the filtered virus. A variety of animals has been given as subject to inoculation with streptococci; namely, rabbits, guinea pigs, dogs, cats, sheep, and monkeys. The streptococci reputed to produce these effects have been cultivated from the throat, tonsils, blood, and cerebrospinal fluid<sup>3</sup> from clinical cases of poliomyelitis, and from the tissues of the central nervous system, cerebral ventricular fluid, intervertebral ganglia, and lymph nodes of cases at autopsy. The most characteristic effect noted in the inoculated animals was paralysis of a flaccid type. This condition is stated to follow various modes of inoculation, but to result most frequently when the cultures are injected into veins.

<sup>1</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1909, liii, 2095. Landsteiner, K., and Levaditi, C., *Compt. rend. Soc. biol.*, 1909, lxvii, 787.

<sup>2</sup> Mathers, G., *J. Am. Med. Assn.*, 1916, lxvii, 1019; *J. Infect. Dis.*, 1917, xx, 113. Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202; *Science*, 1916, xlv, 614; *J. Am. Med. Assn.*, 1917, lxviii, 280. Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205. Nuzum, J. W., *ibid.*, 1916, lxvii, 1437; 1917, lxviii, 24.

<sup>3</sup> Abramson, H. L., *J. Am. Med. Assn.*, 1917, lxviii, 546. The recent report of Abramson on approximately 1,200 fluids from acute cases is pertinent: "Except for a few evident contaminations, our cultures remained sterile."

The findings of these authors conflict in several points with those of previous investigators in this country and abroad. In the first place, the extensive investigations of Flexner and his coworkers, Levaditi and Landsteiner,<sup>4</sup> Leiner and von Wiesner,<sup>5</sup> and Kling, Pettersson, and Wernstedt<sup>6</sup> seemed to exclude ordinary bacteria as the inciting agents of epidemic poliomyelitis. The bacteriological studies carried out at various times and in different countries previously resulted, with one exception to be mentioned, either in obtaining no growths whatever or, as in the case of Geirsvold,<sup>7</sup> of obtaining from the cerebrospinal fluid an ordinary micrococcus which was believed to be a contaminant derived probably from the skin. This does not mean that occasionally bacteria were not cultivated from tissues removed post mortem from human beings and even from inoculated and paralyzed monkeys; but the stricter the conditions of bacteriological asepsis, the less frequently were the cultures obtained. The exception alluded to is the report of the cultivation of a minute, strictly anaerobic organism, globoid bodies so called, by Flexner and Noguchi<sup>8</sup> both from human and monkey tissues.

In the next place, the findings differ essentially with regard to the classes of animals subject to infection with the filterable virus. The only species of animals found to be regularly subject to inoculation are monkeys. Experimental poliomyelitis in the monkey is identical with the disease appearing in man. The clinical symptoms are the same, and the essential lesions in the nervous system indistinguishable. In one unmistakable instance<sup>9</sup> and in an instance not as certainly proven,<sup>10</sup> young rabbits have succumbed to inoculation of nervous tissues carrying the poliomyelitic virus. Neither the symptoms nor the lesions of the nervous system in these animals corresponded with poliomyelitis in man and the monkey.

In still another way are the older and these newer findings in conflict. The recent authors have found streptococci cultivated from a wide variety of tissues and fluids effective on inoculation. Of all the older investigators, only Krause and Meinicke<sup>11</sup> claim to have succeeded in producing infection with blood, cerebrospinal fluid, and various extranevous organs taken from human poliomyelitis.

<sup>4</sup> Levaditi and Landsteiner, *Compt. rend. Soc. biol.*, 1910, lxxviii, 311.

<sup>5</sup> Leiner, K., and von Wiesner, R. R., in Zappert, J., von Wiesner, R. R., and Leiner, K., *Studien über die Heine-Medinsche Krankheit (Poliomyelitis acuta)*, Leipsic and Vienna, 1911, 137.

<sup>6</sup> Kling, C., Pettersson, A., and Wernstedt, W., *Communications Inst. méd. État à Stockholm*, 1912, iii, 5.

<sup>7</sup> Geirsvold, M., *Norsk Mag. Lægevidensk.*, 1905, iii, 1280.

<sup>8</sup> Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

<sup>9</sup> Marks, H. K., *J. Exp. Med.*, 1911, xiv, 116.

<sup>10</sup> Rosenau, M. J., and Havens, L. C., *J. Exp. Med.*, 1916, xxiii, 461.

<sup>11</sup> Krause, P., and Meinicke, E., *Deutsch. med. Woch.*, 1909, xxxv, 1825

It is noteworthy that they also employed rabbits, which they believed to be susceptible to the infection. This work has not been confirmed, except to the extent which the relatively few successful experiments in rabbits by Marks and possibly by Rosenau may be regarded as providing confirmation. But the conditions under which the rabbits succumb are so different from those observed in poliomyelitis that Marks felt obliged to determine the presence of the virus in rabbits by inoculating their nervous tissues into monkeys. The characteristic symptoms and lesions developed. He was able to transmit the virus of poliomyelitis through six series of rabbits. It was then lost.

Finally, the published reports of Mathers, Herzog, and Nuzum, and Rosenow and his coworkers contain descriptions and pictures of lesions occurring in the rabbit and monkey succumbing to the streptococcus infection which they identify with the typical lesions present in poliomyelitis in the central nervous tissues of man and the monkey. This is quite a delicate point to pass upon. Nevertheless it is essential, since the lesions of the nervous tissues in poliomyelitis are particularly characteristic. The descriptions are more convincing than the pictures, which are indeed disappointing. As a matter of fact, on the basis of the published illustrations dealing with lesions in the nervous organs, the suspicion is inevitably aroused that the pathologic processes induced by the streptococci are of another order from those occurring in human cases of poliomyelitis or arising in monkeys from the inoculation of the filtered virus.

We have therefore been led, by the intrinsic importance of the subject, to study streptococci in the manner described by these authors. But we have extended this experimental study to include with streptococci obtained from poliomyelitic patients, streptococci from a variety of other sources.

### *Origin and Properties of the Streptococci.*

*Poliomyelitic.*—Cultures were made from the tonsils of twenty-seven cases of poliomyelitis, and from four pairs of tonsils extirpated during life and one pair removed at autopsy. The cultures were made both from the surface and the interior of the crypts in living cases and from the surface and the interior of the excised organs. The period of the disease at which the cultures were made varied from 3 days after onset to several weeks of convalescence.

*Non-Poliomyelitic.*—The cultures for comparison with the preceding were obtained (a) from the tonsils of children in an institution in which no case of poliomyelitis occurred during the New York epi-



demic (1916), (b) from tonsils extirpated at one of the large clinics of the city,<sup>12</sup> (c) from tonsils excised in private hospitals from adults and children suffering from cardiac or renal disease, and (d) from carious teeth removed because of root infection.

*Cultural Properties.*—The cultures were made on ascitic-fluid-glucose-agar and in ascitic-fluid-glucose-broth. In almost every instance the streptococcus was the predominating organism, in several instances the only organism obtained. No distinction in this respect was observed between poliomyelitic and non-poliomyelitic materials. In every instance subcultures of the second or at most of the third generation yielded pure growths.

The streptococci grew luxuriantly on the media mentioned, and on rabbit blood agar and in rabbit blood broth. The ascitic fluid cultures, after 24 hours' incubation at 37°C., usually contained a sediment at the bottom and upon the sides of the tube, the supernatant fluid being cloudy. The colonies in solid media resemble both those of pneumococcus and of streptococcus. About 1 in 20 of the cultures produced definite zones of hemolysis on the blood agar medium. The rest produced either no, or very slight hemolysis. But they caused a green discoloration of the medium indistinguishable from that produced by typical pneumococci.

The grouping of the organisms was chiefly as diplococci, but short chains were not infrequent. None could be classed as long-chain streptococci. Thirty-nine separate cultures were tested for fermentation. 54 per cent fermented inulin, 90 per cent raffinose, 89 per cent salicin, and 17 per cent mannite. Of these, 8 per cent caused definite, and 13 per cent slight hemolysis.

The morphology was affected by the conditions of cultivation. In rabbit blood broth the forms were quite regular and like streptococci. They became more irregular in the ascitic fluid media. Individuals, often in the same chain, were larger or smaller and more or less elongated. Under strictly anaerobic conditions many individuals appeared of smaller size than in the aerobic cultures.

<sup>12</sup> We are greatly indebted to Dr. Cornelius G. Coakley for many specimens which were removed at his clinic at Bellevue Hospital.

## EXPERIMENTAL.

Practically the same varieties of animals were inoculated as those employed by other recent workers in this field. Intravenous injection was the usual manner of inoculation; but in special instances intracerebral injection, under ether anesthesia, was resorted to. The plan was to inoculate the earliest possible generations. Hence, in 10 per cent of the experiments the first cultivation generation was inoculated, in 70 per cent the second, and in 20 per cent the third generation. Usually the cultures inoculated had been grown in ascitic-fluid-glucose-broth or upon the corresponding agar; in some instances the blood broth or agar cultures were injected.

*Guinea Pigs.*—Twelve guinea pigs ranging in weight from 400 to 500 gm. were injected intravenously with from one-half to two cultures derived from cases of poliomyelitis. In no instance did any signs of infection appear. Hence this species of animals was not employed further.

*Cats.*—Four young animals ranging in weight from 500 to 700 gm. received intravenous injections of one to two cultures. One cat died on the 4th day. No paralysis or other severe symptoms had been detected. The surface of the brain yielded pure cultures of the injected streptococci. Neither brain nor spinal cord showed visible lesions, and sections prepared from both contained no pathologic changes. The remaining three animals remained well.

*Dogs.*—Sixteen young dogs were each given intravenously one-half to one culture per kilo of body weight from cases of poliomyelitis. Of these, one animal developed paralysis, four developed meningitis, and one an arthritis. The other dogs developed no symptoms except vomiting and diarrhea from 1 to 2 hours after inoculation. Two protocols follow.

*Dog 1.*—Weight 2.75 kilos.

Nov. 17, 1916. The animal received intravenously three slant cultures from an acute case of poliomyelitis in the second generation. Nov. 18. Animal weak; does not stand; respiration rapid and shallow. Nov. 19. Left hind leg flaccid; left fore leg weak. Nov. 20. Animal stronger. Paralysis continues. Nov. 21. No change. Nov. 22. Died during night.

*Autopsy.*—The jejunal portion of the intestine was intussuscepted and gangrenous. The meninges of the brain and spinal cord were injected and contained a gelatinous exudate. Stained films showed many streptococci being phagocyted and many free pus cells. A pure culture of streptococci was obtained from the exudate; the heart's blood and other organs gave no growth. Sections of the central nervous organs showed multiple abscesses in the cerebrum, pons, medulla, and white matter of the spinal cord. No lesions characteristic of poliomyelitis were present.

*Dog 2.*—Weight 2.5 kilos.

Nov. 24, 1916. The animal received three slant cultures of a streptococcus in the second generation derived from the tonsil of a poliomyelitic patient on the 3rd day of illness. Nov. 26. Drowsy; does not eat. Nov. 27. Weak; no paralysis. Died at 12 noon.

*Autopsy.*—Except for the central nervous system the organs were normal. The meninges contained an excess of slightly purulent fluid. Films showed pus cells and Gram-negative bacilli but no streptococci. The bacilli were being phagocyted. Only the bacillus was obtained in culture; the heart's blood and meninges yielded no streptococci. The cultural properties of the bacillus placed it in the group of *B. coli*. Sections of the brain and cord showed the existence of acute meningitis, but no lesions of the substance of either.

*Rabbits.*—A much larger number of rabbits was used than of any other species. The reason for this is that rabbits were chiefly used in the work recently reported. The rabbit, is, of course, known to be susceptible to experimental streptococcus and pneumococcus infection, and has been studied with reference to the former more than any other animal. Theoretically, therefore, the course and varieties of streptococcus infection in the rabbit should be well understood. In many ways they are; but this study illustrates the manner in which concentrated attention upon a particular feature of a pathologic process is capable of disclosing a number of unexpected facts. What is vastly more difficult, however, than even this detection is the proper interpretation of the new observations.

In the first place, rabbits alone, of all the varieties of animals studied by us, were inoculated with cultures from other than poliomyelitic sources. 78 animals received intravenous injections of cultures derived chiefly from the tonsils of poliomyelitic patients and 76 received intravenous injections of cultures derived from sources other than poliomyelitis. Table I summarizes the cases of paralysis and menin-

gitis and gives a record of the instances of extranevous infections.<sup>13</sup> A small number of individual protocols follows.

TABLE I.

Paralysis and infections.	Streptococci from poliomye- litic patients.	Streptococci from non-poli- myelitic patients.
Paralysis.....	4	4
Meningitis.....	14	15
Joint infections.....	13	14
Abscess in kidney.....	10	12
Septicemia.....	11	16
Abscess in skeletal muscles.....	5	3
Pericarditis.....	4	3
Endocarditis.....	5	4
Totals.....	66	71

*Streptococci from Poliomyelitic Cases.*

Of the 78 inoculated rabbits, 43 died and 35 still survive. Of the former number 4 developed flaccid paralysis of the limbs (Figs. 1 and 2). 18 other rabbits showed impairment of motion in one or more legs; but in these animals arthritis, tenosynovitis, or general weakness was discovered to be the cause. Daily or twice daily inspection of the animals was found advisable, because a rabbit would frequently show inability to use a leg freely without any apparent lesion, and then a day or two later it would exhibit an acute arthritis in the affected limb, often still present at the autopsy made at a later date.

*Distribution of Lesions in the Rabbits Inoculated with Streptococci from Poliomyelitic Cases.<sup>13</sup>*

Flaccid paralysis.

4

Meningitis.

14

Infection outside nervous organs.

30

Fourteen rabbits developed clinical meningitis to which they succumbed. The symptoms were, as a rule, well marked during life (Fig. 3), and were clearly determined at autopsy (Figs. 5 and 6) and from

<sup>13</sup> Infections in the central nervous system and other organs often occurred in the same animal.

sections of the nervous tissues (Fig. 8), while cultures from the meninges were usually positive. The clinical appearances include opisthotonos, spasticity, and hyperesthesia. In some instances, obvious clinical symptoms were wanting, but a purulent meningitis was found at autopsy. The sections showed at times focal lesions of the central nervous tissues, usually affecting the cerebrum, cerebellum, and medulla, and pons, associated with the meningitis (Figs. 9 and 10).

Thirty rabbits exhibited infections outside the central nervous organs. These consisted most often of arthritis and peri arthritis, but tenosynovitis, myositis, endocarditis, abscess of the kidney, and septicemia were also frequently present (Table I).

A few illustrative protocols will be given. The first is that of a rabbit developing paralysis.

*Rabbit 1.*—Weight 1.5 kilos.

Oct. 13, 1916. The growth from two ascitic-glucose-agar slants from a recent case of poliomyelitis was injected intravenously. Oct. 15. Animal restless. Oct. 16. Head drawn back and to the right; hind legs paralyzed (Fig. 2). Oct. 17. Left fore leg flaccid. 10 a.m. Died.

*Autopsy.*—Outside the nervous system the organs appeared normal except for a small abscess in the left kidney. The meninges were hyperemic, and the cerebellum was covered with a gelatinous exudate. Cultures from the meninges gave a pure growth of streptococcus; the heart's blood gave no growth.

Sections of the brain and cord showed purulent meningitis and slight perivascular extension of the cellular exudate along the pial vessels into the substance of the cerebrum and to a less extent of the spinal cord. The gray matter of the spinal cord and medulla and the intervertebral ganglia were free of any focal lesions. A spinal nerve root in one of the sections showed a purulent infiltration (Fig. 11).

Although this animal showed paralysis of the limbs and to that extent symptoms suggestive of poliomyelitis, lesions of the nervous tissues characteristic of that condition were absent. The only histological changes detected were in the meninges and extensions along the pial investments of the blood vessels into the brain and to a less extent into the spinal cord. What other changes might have been detected had still other portions of the brain and cord been sectioned cannot, of course, be predicted; but as segments from several levels of the spinal cord, from the medulla, pons, and cerebellum, and from



different parts of the cerebrum as well as several intervertebral ganglia were studied, it may be asserted that they would in no way have resembled the typical lesions of poliomyelitis, which are not minutely focal in character.

The question arises as to the immediate causes of the paralysis of the limb. In this instance the source can only be inferred; in others it was found in focal abscesses or necroses, associated with emboli of streptococci, to be described below. Lesions similar to these do not occur in man and the monkey in poliomyelitic infection.

The culture recovered from the meninges of this animal was passed through four other rabbits in succession. The first succumbed in 56 hours to meningitis and septicemia. Sections of the nervous organs showed purulent meningitis and focal abscesses of the brain. The second rabbit, inoculated from the previous one, developed arthritis of both wrist joints. Cultures from this animal produced meningitis in a third, and cultures from the third, septicemia in a fourth rabbit.

Three other rabbits in this series developed, as has been stated, some degree of flaccid paralysis of the limbs. The histological studies of the central nervous organs confirmed essentially the findings given in the protocol above. In each instance there was evidence of acute meningitis with extension of the inflammation along the pial membrane into the substance of the nervous organs and chiefly the cerebrum. The perivascular cellular infiltration always coexisted with and was part of the meningitis; and this in turn was sometimes attended by focal abscesses (Figs. 9 and 10). In none were lesions of the nerve tissues *per se* detected, neither diffuse interstitial infiltration nor degeneration with consequent phagocytosis of the nerve cells. The spinal cord and intervertebral ganglia were notably free of lesions. In rare instances small hemorrhages existed in the spinal cord.

*Rabbit 2.*—Weight 1.55 kilos.

Oct. 10, 1916. The sediment of two tubes of ascitic-glucose-broth containing streptococci in the first generation was injected intravenously. The culture was obtained from the tonsils of a poliomyelitic patient during the first days of illness. Stiffness of the legs was present the following day; that is, in less than 24 hours after inoculation. The chief clinical symptom was spasticity of the hind legs. No further symptoms developed, but death occurred 50 hours after injection.



*Autopsy.*—The thoracic and abdominal organs showed nothing abnormal except that the right kidney contained a small infarcted area. The meninges were hyperemic, but no changes were detected in the dissected brain and cord. No streptococci were cultivated either from the heart's blood or the central nervous organs.

The histological lesions present in the cerebrum consisted of areas of marked perivascular infiltration (Fig. 14) associated with foci of necrosis (Fig. 15). The meninges were the seat of marked infiltration—an acute meningitis. And the relation between the meningitis, perivascular infiltration, and focal necroses was readily established. But the medulla, spinal cord, and intervertebral ganglia were all free of lesions, although the meninges about them showed some degree of cellular infiltration.

In all, five other rabbits of this series showed histological lesions similar to those described. In two of these a purulent meningitis coexisted. In none were any changes present in the gray matter of the spinal cord or in the intervertebral ganglia.

*Rabbit 3.*—Weight 1 kilo.

Nov. 18, 1916. The growth from two ascitic agar slants of a streptococcus isolated from a carious tooth of a convalescent poliomyelitic patient was injected intravenously. Nov. 19. No symptoms had developed. Nov. 20. Pronounced opisthotonos (Fig. 3). Nov. 21. No change. Nov. 22. Meningeal symptoms still present. Rabbit weak. Nov. 23. Condition unchanged. Nov. 24, 8 a.m. Rabbit found dead.

*Autopsy.*—The meninges of the brain and upper portion of the spinal cord were injected. Impression films from the brain and medulla showed many streptococci and pus cells. No lesions were found in the abdominal and thoracic organs. Pure cultures of streptococci were obtained from the surfaces of the brain and medulla.

Sections of the brain and cord showed a purulent meningitis.

The protocols of the rabbits illustrate instances in which (1) flacid paralysis of the limbs developed, (2) spasticity appeared, and (3) meningitis was the prominent symptom. The question arises whether the conditions described correspond to or resemble those present in poliomyelitis as it is known in man and the monkey.

The decision must rest not only upon the gross symptoms but even more upon the histological appearances and in part also upon still another consideration; namely, the possibility of conveying, by inoculation of the rabbit culture material, poliomyelitis to monkeys. This

last aspect of the case will be considered below in connection with the experiments made on monkeys.

In order that the symptoms and lesions in rabbits may be interpreted, it is desirable to recapitulate briefly the main symptoms and lesions of poliomyelitis in man and the monkey. Aside from indefinite signs such as fever, malaise, etc., they consist of meningitis and flaccid paralysis. In man the meningitic symptoms often precede the paralytic, but death appears never to be caused by the meningitis but always by the paralysis, affecting mainly the function of respiration. In the monkey symptoms of meningitis appear, but they are trifling and overshadowed by the paralysis. Death here, too, invariably is caused by the paralysis affecting respiration.

The lesions of the nervous organs of man and the monkey are essentially identical: first, a certain degree of meningeal infiltration, not diffuse, but confined mainly to the region of the large blood vessels, particularly about the medulla and spinal cord and slightly over the cerebrum; second, a marked degree of intramedullary perivascular infiltration affecting slightly the cerebrum, and practically constantly the medulla, spinal cord, and intervertebral ganglia. This perivascular infiltration is associated with more or less diffuse cellular invasion of the gray matter, chiefly of the medulla and spinal cord and rarely of the cerebrum. Focal cellular accumulations also occur in the ganglia, sometimes between, sometimes involving the nerve cells. Hemorrhages are larger and more frequent in man than in the monkey. The nerve cells of the spinal cord, less often of the medulla, and least often of the cerebrum, suffer degeneration and necrosis and subsequent phagocytic destruction. Similar occurrences are observed in the intervertebral ganglia. The nerve cells chiefly affected in the spinal cord are the motor, but the cells in the posterior gray matter also suffer. The lesions in the nervous tissues are not sharply focalized; the cellular invasion of the meninges is chiefly lymphocytic and never chiefly polymorphonuclear or purulent.

The lesions met with in the rabbits are sharply contrasted with these. They consist of meningitis of polymorphonuclear and usually purulent kind, either focal or general; of focal abscesses in the gray matter, most frequent in the cerebrum (Figs. 8, 9, and 10), next in the cerebellum, and very rarely in the medulla and spinal cord; of focal

areas of necrosis of nerve cells, almost exclusively in the cerebrum, surrounded by infiltration of cells of lymphocytic type (Fig. 15) and adjacent to blood vessels showing perivascular infiltration (Fig. 14), extending usually from the meninges. This last and most striking lesion, which may be called focal meningo-encephalitis, is unlike any condition observed in poliomyelitis in man and the monkey. In some instances the foci were general throughout the brain; they were very infrequent in the spinal cord. The distribution of necrotic foci and perivascular infiltration was identical. According to the plane of the section, varying pictures are obtained. Sections through the center (Fig. 15) show a necrotic mass of gray matter surrounded by a collar of mononuclear cells; sections through the periphery may show only a collection of the latter cells (Fig. 13).

Finally, preparations stained for bacteria disclose a wide difference between the findings in man and the monkey and in the rabbits under consideration. While no bacteria have been detected in the former, by the use of ordinary methods, the reverse is true of the latter. The diffuse meningitis in rabbits shows, as a rule, in impression preparations many streptococci and polynuclear leukocytes (Fig. 5). In instances of quickly developing meningitis and rapid death, the cocci are more, and the leukocytes less numerous (Fig. 6). Phagocytosis of the streptococci is common. The focal areas of meningitis also contain streptococci, although not so readily demonstrable. The focal abscesses contain streptococci in masses in the central part and scattered in the periphery (Fig. 7). Probably the central mass was derived from an embolus. In other words, the lesions present in the rabbits are such as would be produced if streptococci or other suitable microorganisms entered the central nervous tissues in such a way and in sufficient numbers to survive and set up infection and inflammation.

This last consideration led to an examination of several stock rabbits of a lot in which snuffles was prevailing. Among three of this group which succumbed to a bacillary septicemia and of which sections of the nervous organs were prepared, one was found showing an acute meningitis attended by perivascular infiltration within the cerebrum (Figs. 12 and 13) indistinguishable from the corresponding conditions present in the animals inoculated with streptococci. In

view of the common prevalence of spontaneous infections among rabbits, one is led to inquire whether the perivascular lesions may not have preexisted in the animals prior to the injection of the streptococci. This supposition has indeed just received strong support from a publication by Reasoner<sup>14</sup> in which he figures this precise lesion as caused by *Spirochæta pallida* localized in the cerebrum of the rabbit. We believe more than ever, therefore, that lesions of this kind, obviously not specific, may be the result of bacillary infection to which rabbits under laboratory conditions are subject. In any case, they cannot be regarded as in any degree peculiar to, and indicative of poliomyelitis, a conclusion supported also by the experiments with streptococci of other than poliomyelitic origin.

*Streptococci from Non-Poliomyelitic Cases.*

76 rabbits were inoculated with streptococci of non-poliomyelitic origin. Of these, 58 rabbits died in the course of the experiments. No differences could be detected between the effects of these streptococci and those obtained from cases of poliomyelitis. Even the numerical results are equivalent.

*Distribution of Lesions in the Rabbits Inoculated with Streptococci from Non-Poliomyelitic Cases*<sup>13</sup>

Flaccid paralysis.	Meningitis.	Infections outside nervous organs.
4	15	37

A few illustrative protocols follow.

*Rabbit 4. Spastic, Succeeded by Flaccid Paralysis.*—Weight 2 kilos.

Oct. 16, 1916. Two slant cultures were injected intravenously. The streptococcus employed was in the second generation and came from the extirpated tonsils of an adult suffering from chronic nephritis. Oct. 20. Sluggish. Oct. 21. Hind legs spastic. Oct. 22. Hind legs flaccid; animal prostrate; unable to rise. 6 hours later marked opisthotonos appeared, and 1½ hours afterwards death occurred.

*Autopsy.*—Except for the central nervous system, the internal organs appeared normal. The muscles about the hip joints contained abscesses from which streptococci were recovered in films and cultures. The meninges of the medulla

<sup>14</sup> Reasoner, M. A., *J. Am. Med. Assn.*, 1916, lxvii, 1799.

and cerebellum contained a gelatinous exudate from which, as well as from the fourth ventricle, streptococci were found in films and cultivated.

Sections from all the nervous organs showed (1) in the cerebrum and cerebellum multiple focal abscesses containing large numbers of streptococci; (2) focal purulent meningitis, in which streptococci were difficult to demonstrate; (3) the absence of lesions in the medulla, spinal cord, and intervertebral ganglia.

*Rabbit 5. Flaccid Paralysis and Convulsions.*—Weight 1.8 kilos.

Nov. 2, 1916. Three slant cultures were injected intravenously. The streptococcus employed was in the second generation and had been obtained from the tonsil of a child in an isolated infant home in which no case of poliomyelitis had developed. Nov. 4. Left fore leg flaccid (Fig. 4); no other symptoms. Nov. 9. No change until today. About 1 p.m. convulsions developed which became more and more violent. 2.20 p.m. Died.

*Autopsy.*—The abdominal and thoracic organs appeared normal. The meninges of the brain and cord were moderately congested, and an exudate was present in the lumbar region. Dissection revealed nothing. The joints and muscles of the paralyzed leg showed no abnormality. Cultures and films from the blood, nervous organs, and joints showed no streptococci.

Sections prepared from various portions of the nervous organs showed merely punctiform hemorrhages in the cervical cord, medulla, and pons.

*Rabbit 6. No Characteristic Symptoms. Perivascular Lesions in the Meninges and Cerebrum.*—Weight 1.6 kilos.

Nov. 6, 1916. Two slant cultures in the second generation were injected intravenously. The streptococci were obtained from the extirpated tonsils of an otherwise healthy child. Nov. 7. No symptoms. Nov. 8. Died.

*Autopsy.*—The surface of the kidneys contained punctiform hemorrhages; spleen enlarged; acute pericarditis. The meninges were highly congested and an exudate covered the cerebellum and medulla. Streptococci and polymorphonuclear leukocytes were present in the exudate in the pericardium and meninges. Cultures were positive.

Sections from the nervous organs showed infiltration of the meninges and about the blood vessels (Fig. 16) in the cerebrum, attended by foci of necrosis (Fig. 17).

A second rabbit inoculated with this strain developed fatal septicemia.

After what has already been said in the discussion of the results of the injection of rabbits with streptococci derived from cases of poliomyelitis, little need be added here. The essential facts seem to be (1) that streptococci derived from conditions other than poliomyelitis are capable of producing lesions and symptoms in rabbits indistinguishable from those produced by streptococci of poliomyelitic



origin; and (2) that with a sufficiently large series of rabbits, the numerical proportion of lesions tends to be identical in both instances. The general conclusion indicated by the two classes of experiments is that when large numbers of streptococci possessing a certain grade of virulence are injected into the veins of rabbits, they tend by preference to localize in the meninges, from which the cerebrum may subsequently be invaded; next to the meninges, they seek the joints, and then the voluntary muscles and other situations. In these several localizations, they set up inflammations and suppurations in which they survive for a time, but they may also die. The conditions are analogous to experimental pneumococcus infection in the dog. This animal, relatively resistant to pneumococci, will respond to very large doses by the development of a purulent meningitis and associated encephalitis.<sup>15</sup> In the rabbit, too virulent cultures quickly kill through septicemia unassociated with particular localizations, while avirulent cultures are quickly destroyed, and hence do not localize at all.

In other words, it is not evident that the nervous lesions of streptococcus infection in the rabbit can be identified with the specific disease poliomyelitis as we know its manifestations in man and the monkey.

#### *Experiments on Monkeys.*

In this paper the monkey has frequently been referred to as the one experimental animal in which thus far the typical symptoms and pathologic lesions of poliomyelitis in man have been reproduced. Hitherto, infection of the monkey has been accomplished by means of the filterable virus contained within the nervous and some other organs of human beings suffering from poliomyelitis, and in a few instances besides through inoculation of the anaerobic cultivated globoid bodies of Flexner and Noguchi.<sup>8</sup> The recent workers alluded to<sup>2</sup> claim to have produced typical poliomyelitis in the monkey with streptococci. Since this species supplies the decisive test, we carried out crucial experiments with it.

The method of inoculation recommended by these authors departs considerably from the one usually employed with the filterable virus.

<sup>15</sup> Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.



With the latter, infection by way of the blood is difficult or impossible to achieve;<sup>16</sup> with the streptococci, intravenous inoculation has been preferred.

*Monkey 1.*—Nov. 18, 1916. A *Macacus rhesus* was given intravenously a suspended sediment from 60 cc. of ascitic-fluid-glucose-broth culture in the second generation of streptococcus obtained from the tonsils from a poliomyelitic patient. Nov. 19. Animal moves about more slowly; generally weak; no paralysis. Nov. 20, 8 a.m. Voice high pitched. 10 a.m. Legs spastic. 11 a.m. Retraction of head; marked hyperesthesia. 1 p.m. Died.

*Autopsy.*—The organs outside the nervous system showed nothing of importance. The meninges were congested and edematous. The substance of the brain and cord appeared normal. Films from the meninges of the brain and cord showed streptococci, pus cells, and mononuclear cells; pure cultures of streptococcus were obtained.

Sections of the brain and cord showed only a purulent meningitis. No lesions were detected in the substance of the spinal cord, medulla, pons, cerebrum, or intervertebral ganglia.

*Monkey 2.*—Dec. 11, 1916. A *Macacus rhesus* was given intravenously the suspended sediment from 60 cc. of ascitic-glucose-broth culture. The culture of streptococcus used in this experiment came from the tonsil of a poliomyelitic patient and had been passed through two rabbits, of which one had developed paralysis of the legs, and both at autopsy had shown purulent meningitis and focal cerebral abscesses. Dec. 12. No general effect; double panophthalmitis appearing. Dec. 13. No change in the general condition; anterior chamber and vitreous opaque and purulent. The sediment from 30 cc. of a similar culture was injected intravenously. Within 2 hours the animal became ill and weak; no paralysis was detected. Dec. 14. Died.

*Autopsy.*—The lungs were edematous and hemorrhagic; spleen swollen, dark, and friable; mesenteric nodes hemorrhagic. The meninges were congested; there was no visible lesion of the spinal cord or brain aside from punctate hemorrhages in the latter. Films and cultures prepared from the interior of the eyes and the fourth ventricle gave streptococci. Cultures from the heart's blood and surface of the brain were negative.

Sections of the brain, medulla, spinal cord, and intervertebral ganglia showed no lesions aside from small extravasations of blood in the cerebrum.

Since this is the type of experiment which is said by Rosenow and his coworkers to have given the most constant result, it was repeated on four other *Macacus rhesus* monkeys. In each instance some degree of depression followed the massive injections; in one instance a

<sup>16</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

purulent panophthalmitis again developed. Paralysis never appeared, and all four animals recovered.

In other words, our experiments were uniform failures as far as producing in monkeys paralysis or any condition resembling poliomyelitis was concerned, by means of cultures of streptococci derived from cases of poliomyelitis and injected either directly into monkeys or first into rabbits and then into monkeys. As will be shown below, the monkeys which received massive doses of the streptococci showed no undue resistance to inoculation with the filtered virus of poliomyelitis.

#### *Streptococci from the Poliomyelitic Spinal Cord.*

The attention which has been directed to streptococci in poliomyelitis has led to the discovery that streptococci can be cultivated from specimens of the brain and spinal cord apparently of both persons and monkeys succumbing to the disease. By employing considerable portions of the spinal cord of monkeys, we have in a few instances obtained growths of streptococci from them. Only a part of the cultures made yield the cocci; many tubes remain sterile. The following protocol illustrates this point.

When moribund, a poliomyelitic monkey was etherized and portions of the brain and spinal cord and two lymph nodes were removed aseptically. The brain and cord were inoculated (*a*) in fragments and (*b*) in emulsions into ascitic-glucose-broth and agar respectively. The lymph nodes were inoculated into the fluid medium. The agar medium was inoculated while fluid and then allowed to congeal. Twenty tubes in all were inoculated. One-half the tubes were covered with paraffin oil. The incubation was at 37°C. After 48 hours two broth tubes containing emulsified brain tissue contained growths of streptococci. After 72 hours four more tubes of broth showed streptococci, of which two had been inoculated with brain emulsion, one with a brain fragment, and one with a lymph node. 14 tubes remained sterile. It is worth noting that the comminuted brain tissue alone, except for one brain fragment, yielded streptococci; the spinal cord cultures yielded none. The distribution of the streptococci in the central nervous system must at best have been quite irregular. Moreover, none of the solid cultures yielded detectable growths. A single surviving streptococcus in a fluid culture would multiply abundantly; in a solid culture it would probably be lost. The streptococci from these sources were injected intravenously into six medium sized rabbits. One rabbit died in 48 hours. The

autopsy showed a purulent meningitis. Another developed arthritis 72 hours after inoculation. The remaining four showed no symptoms.

The original streptococcus from this source was transferred to 60 cc. of ascitic-glucose-broth of which, after incubation at 37°C., the sediment was injected intravenously into a *Macacus rhesus*. No symptoms developed, and hence 48 hours later, the sediment from 30 cc. of a broth culture was also injected. Within 24 hours the animal was somewhat slow and irritable; the condition quickly passed away. No paralysis or other severe symptoms developed.

At the same time that the ascitic broth culture was prepared, another was made from the original growth in blood agar. The entire growth was washed off and injected intracerebrally, under ether anesthesia, into a *Macacus rhesus*. No symptoms of any kind developed.

The streptococcus derived from the brain tissue produced a green color in blood and otherwise resembled closely *Streptococcus viridans*, so called.

Before considering the significance of this experiment, the following additional note should be made. Several stock specimens of recently glycerolated spinal cord from monkeys which had succumbed to poliomyelitis were incubated in ascitic-glucose-broth and a specimen was obtained yielding streptococci corresponding to *Streptococcus viridans*. Recent cultures from this source were inoculated (a) intravenously into two rabbits, (b) intracerebrally, under ether anesthesia, into a *Macacus rhesus*, and (c) the latter test was controlled by inoculating a filtrate of the spinal cord itself into another *Macacus rhesus*. The results are significant. The rabbits each developed meningitis and kidney abscesses, without paralysis. Sections of the spinal cord and brain showed only lesions of meningitis. The monkey inoculated with the streptococcus remained well; the other inoculated with the filtrate developed paralysis and died.

The conditions presented by the foregoing experiments on monkeys should theoretically, assuming that streptococci have an etiologic relation to poliomyelitis, have been favorable to the production of paralysis and the characteristic nervous lesions in at least part of the monkeys inoculated. That in no single instance any definite infection resulted except purulent ophthalmitis, and that the streptococci derived immediately from the nervous tissues of poliomyelitic monkeys should have been inert is difficult to harmonize with the view that the streptococcus is capable of producing poliomyelitis in monkeys, especially as the filtrate from the nervous tissue of the paralyzed monkey was readily effective under circumstances in which the streptococcus obtained from the same source was entirely inactive.

*Does Streptococcus Infection Protect against Infection with the Filtered Virus?*

Recovery from poliomyelitis induced by the filtered virus leaves a state of resistance in monkeys indicated by insusceptibility to reinoculation<sup>17</sup> and by the power of the blood serum to neutralize the filtered virus.<sup>18</sup> Tests were made to determine whether monkeys having received several injections of cultures of streptococci from poliomyelitic sources exhibited protection by either of the foregoing reactions.

*Neutralization in Vitro.*—For this test two monkeys which had resisted streptococcus inoculation were taken and their immunity to the organism was increased by the following injections: to each was given intravenously the sediment from 100 cc. of broth culture of the original strain employed and then on 2 successive days sediment from 10 cc. of a broth culture of the streptococcus cultivated from the glycerolated spinal cord of a poliomyelitic monkey. 10 days after the last injection the sera of the monkeys agglutinated the streptococci in dilutions up to 1:1,500. Normal monkey serum agglutinated the same cultures up to 1:300.

The tests for neutralization were made by mixing 0.1 cc. of a Berkefeld filtrate prepared from a 5 per cent emulsion of active poliomyelitic virus with 1 cc. of monkey serum, incubating for 2 hours at 37°C., and keeping for 16 hours at 4°C. The injections were made intracerebrally, under ether anesthesia, into two *Macacus rhesus* monkeys.

*Monkey 3.*—Jan. 3, 1917. A *Macacus rhesus* was inoculated. No symptoms appeared until Jan. 10 when the right arm became flaccid. Jan. 11. The left arm was flaccid. Jan. 12. The muscles of the trunk were paralyzed. Jan. 13. The animal was etherized.

*Autopsy.*—The gross poliomyelitic lesions of the medulla were typical, and the sections of the spinal cord, medulla, and intervertebral ganglia were characteristic of the disease.

*Monkey 4.*—Jan. 3, 1917. A *Macacus rhesus* was inoculated. No symptoms developed until Jan. 7 when the animal protected the right arm. Jan. 11. Ataxia

<sup>17</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 45.

<sup>18</sup> Landsteiner and Levaditi, *Compt. rend. Soc. biol.*, 1910, lxxviii, 311.

and pronounced tremor of head. Jan. 12. Legs flaccid; animal prostrate. Jan. 13. Etherized.

The spinal cord and medulla showed typical gross lesions and the sections were characteristic of poliomyelitis.

*Protection Tests.*—Three monkeys which had received large injections of streptococci from cases of poliomyelitis were tested for protection against the filtered virus.

*Monkey 5.*—Nov. 22, 1916. A *Macacus rhesus* received intravenously 50 cc. of ascitic-glucose-broth culture of poliomyelitic streptococci which had been passed through one rabbit. A period of depression followed, but on Nov. 24 the animal appeared normal. The streptococci from 40 cc. of a similar broth culture were injected. No marked symptoms developed. Dec. 12. 1 cc. of the filterable virus from fresh brain and spinal cord of a paralyzed monkey was injected intracerebrally under ether anesthesia. Dec. 17. Ataxia. Dec. 18. Prostrate. Dec. 19. Died.

The autopsy showed marked poliomyelitic lesions.

*Monkey 6.*—Nov. 26, 1916. A *Macacus rhesus* received intravenously 45 cc. of a broth culture of streptococcus of poliomyelitic origin. Nov. 28. No symptoms had appeared. The sediment from 50 cc. of culture was injected. Dec. 1. Animal weak, moves about slowly. Dec. 3. Recovered. Dec. 12. Very active. 1 cc. of a suspension of brain and spinal cord of poliomyelitic monkeys was injected intracerebrally under ether anesthesia. Dec. 15. Ataxia; tremor; double ptosis. Dec. 17. Prostrate. Dec. 21. Very weak. Etherized.

The autopsy showed typical lesions of poliomyelitis.

*Monkey 7.*—Dec. 6, 1916. A *Macacus rhesus* received intravenously the sediment from 60 cc. of an ascitic-glucose-broth culture of poliomyelitic streptococci, Dec. 7. Animal somewhat ill; eyes cloudy. Dec. 8. The sediment from 30 cc. of a culture was injected intravenously. Dec. 9. Moves slowly; not well. Dec. 12. Well. Jan. 23, 1917. The sediment from 100 cc. of a broth culture was injected. No effect. Jan. 24. 10 cc. of a broth culture of streptococcus obtained from the nervous tissues of a poliomyelitic monkey were injected. Jan. 25. 10 cc. of the same culture were injected as on the day before. No effect produced. Feb. 3. 0.5 cc. of a suspension of poliomyelitic spinal cord of a monkey was injected intracerebrally under ether anesthesia. Feb. 7. Both arms paralyzed. Feb. 8. Prostrate. Etherized.

The spinal cord showed marked lesions of poliomyelitis.

#### DISCUSSION.

The results obtained by us from the inoculation of several species of laboratory animals with cultures of streptococci from poliomyelitic patients and other sources fail to establish an etiologic relationship



between streptococci and epidemic poliomyelitis. In this respect our experiments are in sharp contrast to those of Mathers, Herzog, and Nuzum, and Rosenow and his coworkers. Not only were we unsuccessful in inciting poliomyelitis with cultures of streptococci, but we found that these organisms produce chiefly in rabbits various conditions and lesions that are absent from man and the monkey, the subjects of poliomyelitis, and the conditions and lesions result equally well from streptococci originating in persons not suffering from or exposed to the disease. In other words, they are referable not to poliomyelitis, but to streptococcus infection. That they may also be present accidentally in stock rabbits has also been shown. Finally, we have failed to detect any immunological relationship between streptococci and the filterable virus of poliomyelitis.

It may be pointed out that the mere appearance of paralysis in animals is no sufficient criterion for concluding that poliomyelitis exists or has been produced. The toxin of the Shiga bacillus, for example, frequently causes flaccid paralysis in rabbits. It may be considered as established that streptococci do sometimes cause paralysis in rabbits and possibly other animals, but our experiments show that this is determined not by the source but by the degree of virulence of the cultures. The final test indeed is the monkey, not the rabbit or other domestic animals, and we not only failed with streptococci to induce paralysis in monkeys, but we failed to render them more resistant or their blood serum neutralizing to the filtered virus of poliomyelitis.

Our failures cannot be ascribed to minor variations in technique. About 10 per cent of the streptococcus cultures from poliomyelitic cases were obtained pure in the first generation and at once inoculated; and no distinction was discovered between the lesions thus produced and those set up by the cultures in the second and third generations. There is one advantage which the earlier generations possess over the later ones; they are somewhat more virulent. Yet even the first generations possessed little infective power and enormous doses were required to produce any pathologic effect. The rapid loss of power of streptococci to produce paralysis even when passed from animal to animal is in sharp contrast with the increase occurring under similar conditions in the filtered virus. No two sets



of pathologic actions and effects could be more diverse than those observed by us to be caused by streptococci and by the filtered virus of poliomyelitis.

#### SUMMARY.

Streptococci cultivated from the tonsils of thirty-two cases of poliomyelitis were used to inoculate various laboratory animals.

In no case was a condition induced resembling poliomyelitis clinically or pathologically in guinea pigs, dogs, cats, rabbits, or monkeys.

On the other hand, a considerable percentage of the rabbits and a smaller percentage of some of the other animals developed lesions due to streptococci. These lesions consisted of meningitis, meningo-encephalitis, abscess of the brain, arthritis, tenosynovitis, myositis, abscess of the kidney, endocarditis, pericarditis, and neuritis.<sup>19</sup>

No distinction in the character or frequency of the lesions could be determined between the streptococci derived from poliomyelitic patients and from other sources.

Streptococci isolated from the poliomyelitic brain and spinal cord of monkeys which succumbed to inoculation with the filtered virus failed to induce in monkeys any paralysis or the characteristic histological changes of poliomyelitis. These streptococci are regarded as secondary bacterial invaders of the nervous organs.

Monkeys which have recovered from infection with streptococci derived from cases of poliomyelitis are not protected from infection with the filtered virus, and their blood does not neutralize the filtered virus *in vitro*.

We have failed to detect any etiologic or pathologic relationship between streptococci and epidemic poliomyelitis in man or true experimental poliomyelitis in the monkey.

<sup>19</sup> Many thousand cases of epidemic poliomyelitis occurred in New York and elsewhere during the past summer (1916) and many cases were subjected to autopsy. But no instance of metastatic infection and inflammation such as occur usually in streptococcus infection has been reported.

## EXPLANATION OF PLATES.

## PLATE 47.

FIG. 1. Rabbit. Flaccid paralysis of fore leg appearing 48 hours after an intravenous inoculation of streptococci from a poliomyelitic tonsil.

FIG. 2. Rabbit. Flaccid paralysis of both hind legs and meningitis appearing 72 hours after an intravenous inoculation of poliomyelitic streptococci.

## PLATE 48.

FIG. 3. Rabbit. Meningitis and opisthotonos appearing 48 hours after an intravenous inoculation of poliomyelitic streptococci.

FIG. 4. Rabbit. Flaccid paralysis of the left fore leg appearing 72 hours after an intravenous inoculation of non-poliomyelitic streptococci.

## PLATE 49.

FIG. 5. Contact film from the cerebrum of the rabbit shown in Fig. 2.  $\times 1,000$ .

FIG. 6. Contact film from the cerebrum of a rabbit which survived 72 hours after an intravenous inoculation of non-poliomyelitic streptococci.  $\times 1,000$ .

FIG. 7. Streptococci in brain abscesses from a rabbit which survived 72 hours after an intravenous inoculation of poliomyelitic streptococci. Gram-Weigert stain.  $\times 1,000$ .

## PLATE 50.

FIG. 8. Purulent meningitis. Section from the rabbit shown in Fig. 2.  $\times 250$ .

FIG. 9. Cerebral abscess. Section from a rabbit which survived 72 hours after an intravenous inoculation of non-poliomyelitic streptococci from the tonsil of an adult.  $\times 250$ .

FIG. 10. Cerebellar abscesses from the same rabbit as Fig. 9.  $\times 40$ .

## PLATE 51.

FIG. 11. Purulent neuritis. Section from a spinal nerve root of a rabbit which survived 72 hours after an intravenous inoculation of poliomyelitic streptococci.  $\times 250$ .

FIG. 12. Perivascular infiltration. Cerebrum of a stock rabbit which succumbed to bacillary infection.  $\times 250$ .

FIG. 13. Round cell infiltration from the same rabbit as Fig. 12.  $\times 250$ .

## PLATE 52.

FIG. 14. Perivascular infiltration. Cerebral cortex of a rabbit which survived 4 days after an intravenous inoculation of poliomyelitic streptococci.  $\times 250$ .

FIG. 15. Focal necrosis and round cell infiltration. Cerebrum of a rabbit which survived 7 days after an intravenous inoculation of poliomyelitic streptococci.  $\times 180$ .

## PLATE 53.

FIG. 16. Perivascular infiltration. Cerebrum of a rabbit which survived 48 hours after an intravenous inoculation of non-poliomyelitic streptococci.  $\times 105$ .

FIG. 17. Focal necrosis and round cell infiltration. Cerebrum of the same rabbit as Fig. 16.  $\times 105$ .



FIG. 1.



FIG. 2.

(Bull: Streptococci from Poliomyelitis.)



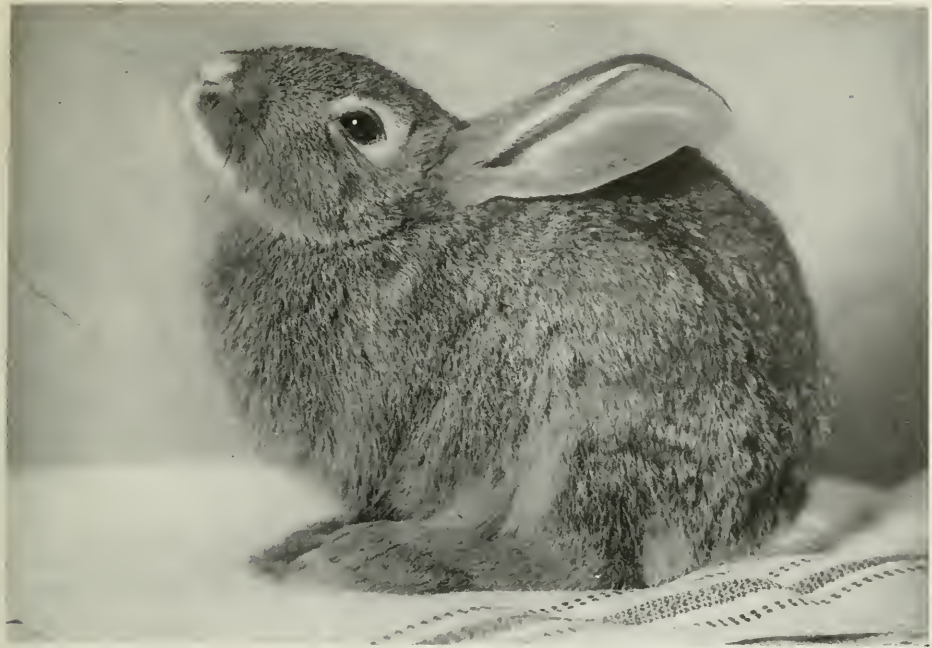


FIG. 3.



FIG. 4.

(Bull: Streptococci from Poliomyelitis.)





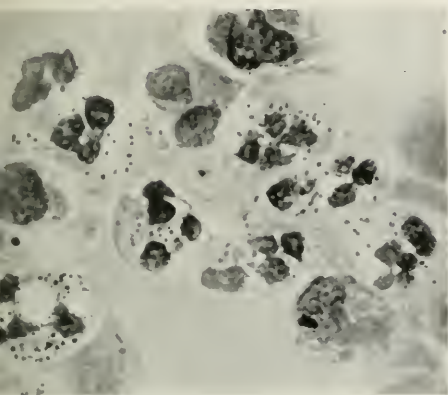


FIG. 5.

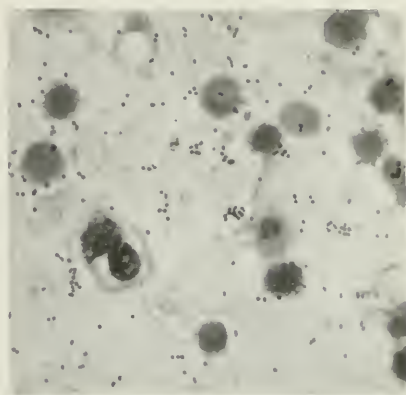


FIG. 6.



FIG. 7.

(Bull: Streptococci from Poliomyelitis.)



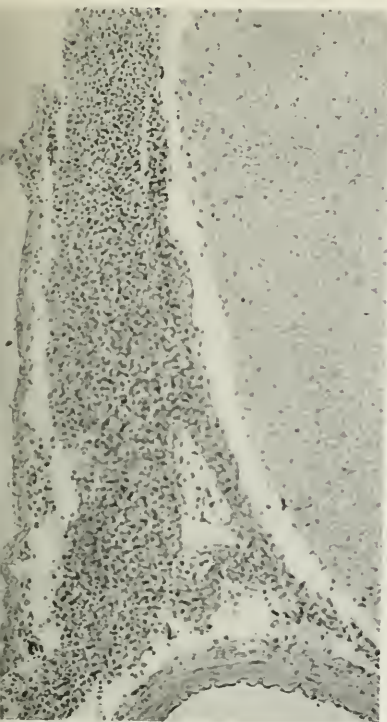


FIG. 8.

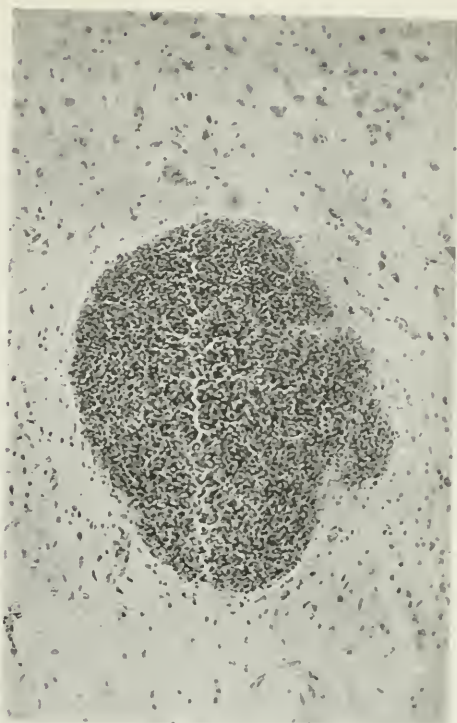


FIG. 9.

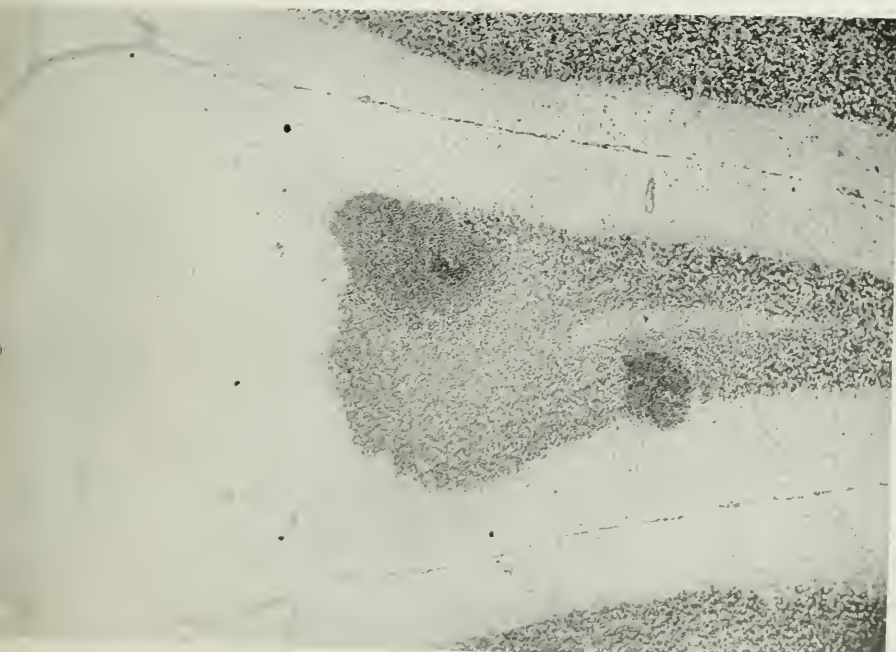


FIG. 10.

(Bull: Streptococci from Poliomyelitis.)







FIG. 11.

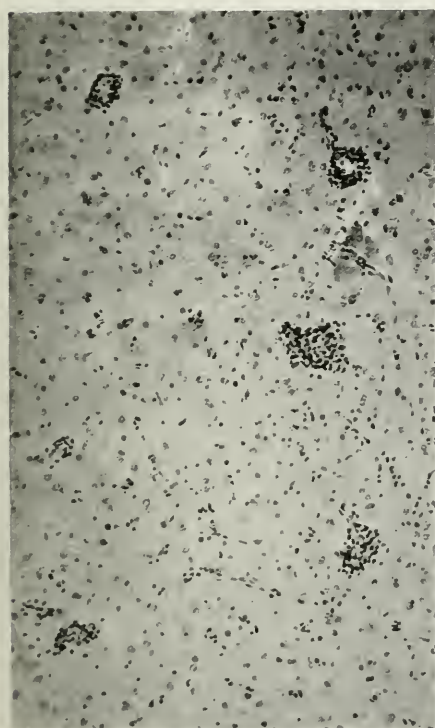


FIG. 12.

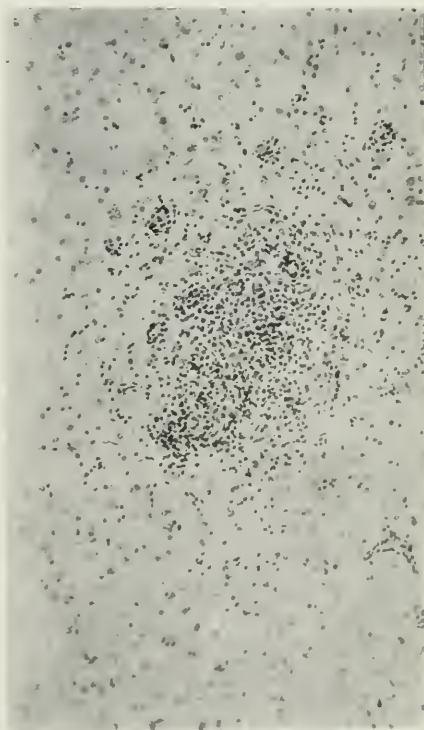


FIG. 13.

(Bull: Streptococci from Poliomyelitis.)





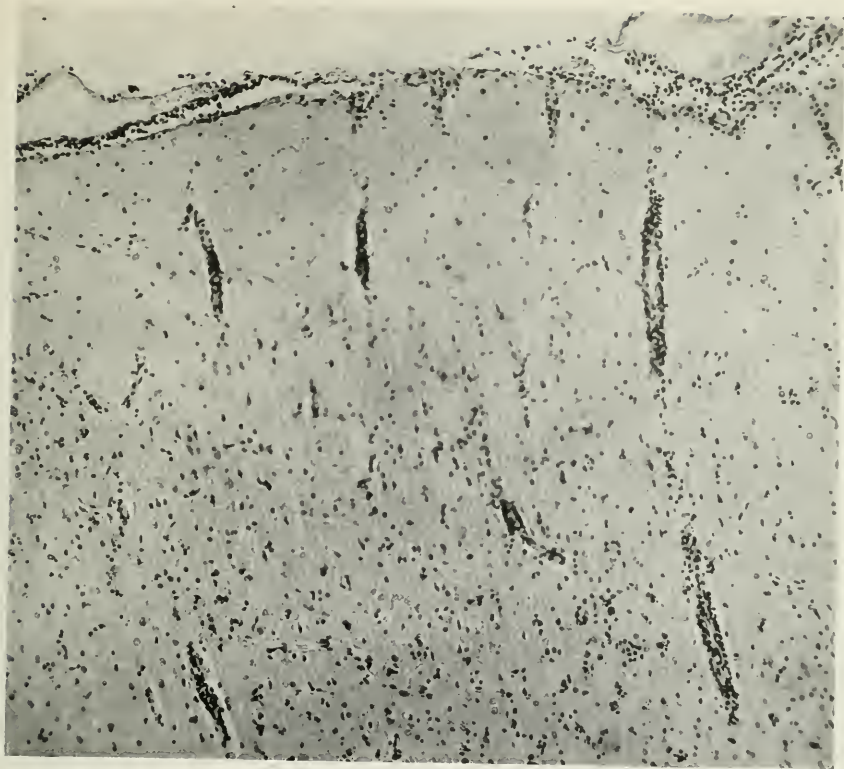


FIG. 14.

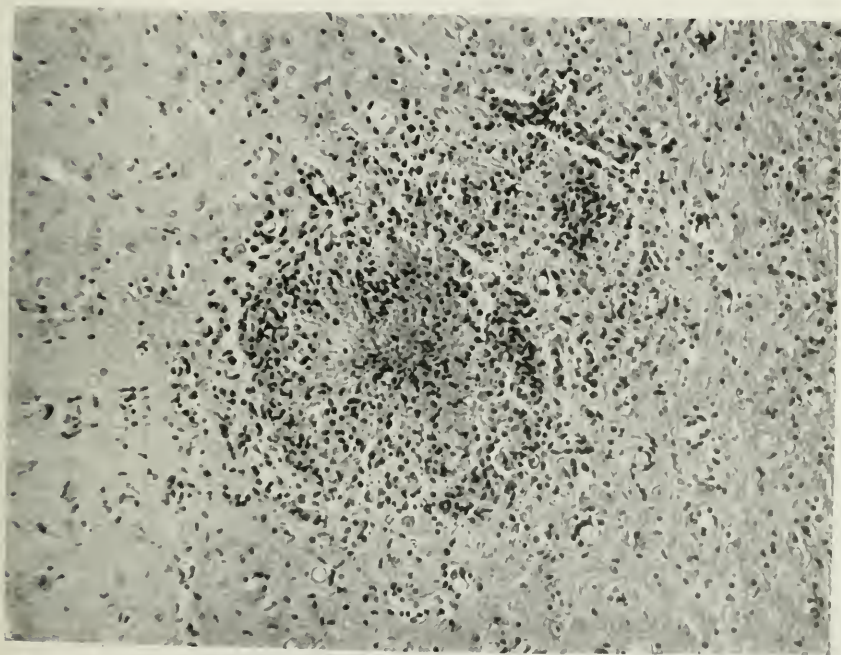


FIG. 15.

(Bull: Streptococci from Poliomyelitis.)



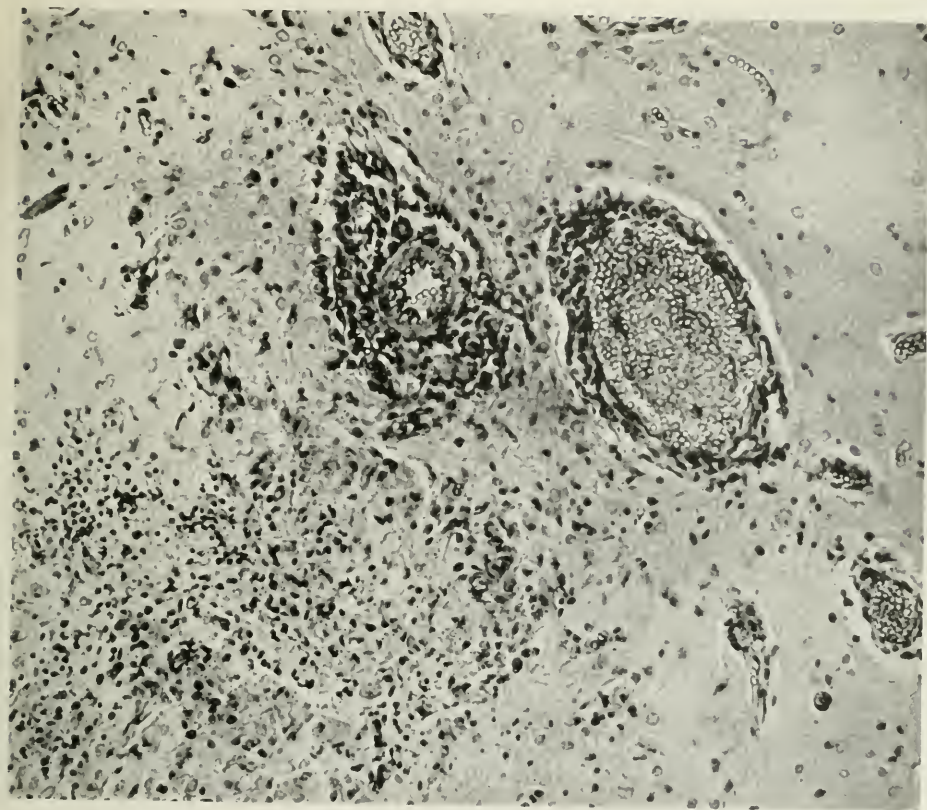


FIG. 16.

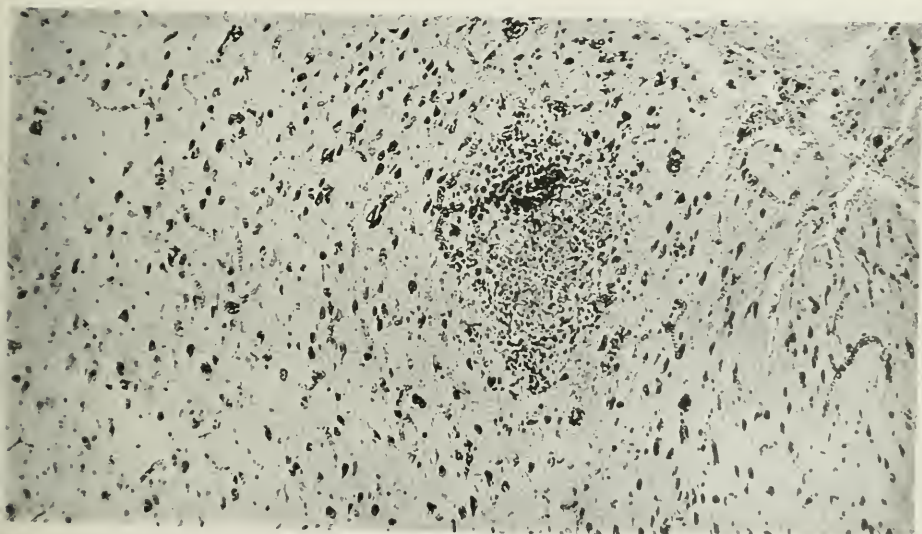


FIG. 17.

(Bull: Streptococci from Poliomyelitis.)





# A REPORT ON THE SERUM TREATMENT OF TWENTY-SIX CASES OF EPIDEMIC POLIOMYELITIS.

By HAROLD L. AMOSS, M.D., AND ALAN M. CHESNEY, M.D.

(From The Rockefeller Institute for Medical Research, New York, and the Westchester County Isolation Hospital, New York State Board of Health.)

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## INTRODUCTION.

This report describes in some detail the method used and the results attained in twenty-six cases of acute poliomyelitis treated with human serum from recovered and convalescent cases of the disease. The cases were treated during the epidemic which prevailed in the summer and autumn of 1916 in New York State.

The epidemic was attended by a high death rate uniformly affecting large groups of cases occurring in city and suburban localities. In this, as in previous epidemics, the greater number of deaths occurred in those cases in which the upper portions of the spinal cord were involved, or in which an ascending, or Landry's type of paralysis occurred. The percentage of deaths from secondary causes, such as pneumonia, was small. Aside from this, no secondary or metastatic foci of infection, outside the central nervous organs, arose or were detected. This fact should be mentioned in connection with the claims that have been made that epidemic poliomyelitis is a form of streptococcus infection.<sup>1</sup>

At the onset the disease may offer a perplexing problem to the attending physician or expert diagnostician. In both the field and ward work at the Westchester Isolation Hospital use was made of lumbar puncture and microscopic and chemical examinations of the cerebrospinal fluid at the bedside in order to arrive at a rapid differential

<sup>1</sup> Mathers, G., *J. Infect. Dis.*, 1917, xx, 113. Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202. Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205.



diagnosis. In addition to detecting two cases of epidemic cerebrospinal meningitis which quickly yielded to injections of the antimeningococcic serum, and one case of acute meningitis due to a bacillus probably belonging to the paracolony group, the procedure commended itself especially as it enabled us, first, to remove to the hospital cases on the 1st or 2nd day of illness, thereby contributing materially to the efficiency of the isolation, and, next, to bring under the specific serum treatment cases in the earlier stages of the affection. The groups of histories given show the advantages of the latter point, but it is mentioned here since it has been proved feasible, in field work in country districts, to take the necessary apparatus to the bedside and to make a diagnosis immediately by means of lumbar puncture and clinical examinations. Valuable time may be lost if the fluid is sent to the laboratory, and in the most perplexing cases the diagnosis can be made only by considering the character of the cerebrospinal fluid, as revealed by microscopic and chemical examination, in connection with the clinical findings at the time the fluid is withdrawn. In some cases, though rarely, it may be necessary to repeat the lumbar puncture in 6 hours.

#### HISTORICAL.

The discovery, almost simultaneously by Flexner and Lewis<sup>2</sup> and Landsteiner and Levaditi,<sup>3</sup> of the filterable nature of the microorganism, or virus, causing the disease, was quickly followed by Flexner and Lewis's<sup>4</sup> observation that recovery from an attack of experimental poliomyelitis afforded protection to a second inoculation; and this in turn was followed by the detection of immunity or neutralizing substances in the blood serum first of recovered monkeys and then of recovered human beings.<sup>5</sup> Since recovery from an attack of poliomyelitis was obviously brought about through a process of immunization, similar to that in other infectious diseases, Flexner and Lewis<sup>6</sup> endeavored to prevent the development of the

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<sup>2</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1909, liii, 2095.

<sup>3</sup> Landsteiner, K., and Levaditi, C., *Compt. rend. Soc. biol.*, 1909, lxxvii, 592.

<sup>4</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 45.

<sup>5</sup> Levaditi and Landsteiner, *Compt. rend. Soc. biol.*, 1910, lxxviii, 311. Römer, P. H., and Joseph, K., *Münch. med. Woch.*, 1910, lvii, 568. Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1780. Anderson, J. F., and Frost, W. H., *J. Am. Med. Assn.*, 1911, lvi, 663.

<sup>6</sup> Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1780.

infection in inoculated monkeys through the administration of blood serum taken (a) from recovered monkeys and (b) from recovered human beings. The results, while not constant and regular, were definite. The method pursued by Flexner and Lewis was first to make an intracerebral inoculation of the active adapted monkey virus, and then, about 24 hours later, to begin treatment by intraspinal injections of the immune serum. Their results were prevention of the onset of paralysis in some cases, and delay of the onset in others. Account should be taken, however, of the high potency of the adapted virus and the mode of its inoculation since the number of infections following was 100 per cent and the mortality among the paralyzed animals nearly as high. The experimental disease is, therefore, far more severe than the corresponding epidemic condition in man; and presumably it is less subject to favorable influence through therapeutic measures.

These experimental results were at once utilized as a basis of a serum therapy in man.<sup>7</sup> Netter and his associates<sup>8</sup> have reported a total of thirty-four cases of acute poliomyelitis which they have treated by the subdural method of injecting immune serum. They have undoubtedly established the fact that, as in the monkey, subdural injections intelligently carried out in man are safe. They believe further that they have proved them to be definitely beneficial or curative. They became increasingly convinced that the period of the disease at which the injections were made counted vitally, and they urged that the injections should be made as early as possible in the course of the infection.

During the past summer the serum treatment has been carried out in a number of different hospitals. Very few reports giving the circumstances and details of the methods and results have thus far appeared. But a number of important facts bearing on the subject have been acquired from clinical observation and experimental study. In the first place, a meningitis accompanied by a pleocytosis precedes in many instances the onset of paralysis. It is, therefore, one of the particular tasks of the attending physician to detect the disease in this early period when the treatment offers the greatest possibility of benefit. In the second place, the meningitis is attended by an increased permeability of blood vessels in the inflamed pia arachnoid. By reason of this increased permeability, it is possible for protein and other substances introduced into the blood to reach the subarachnoid spaces and probably the perilymphatic spaces in the nervous organs

<sup>7</sup> Netter, A., Gendron, A., and Touraine, *Compt. rend. Soc. biol.*, 1911, lxx, 625.

<sup>8</sup> Netter, A., *Bull. Acad. méd.*, 1915, lxxiv, series 3, 403. Netter, A., and Salanier, M., *Bull. et mém. Soc. méd. hôp. Paris*, 1916, xl, series 3, 299.

which are otherwise excluded. Hence, while the most direct route to the perivascular lymphatic and perineural spaces in the spinal cord may still be by way of the cerebrospinal fluid, this is not the only way since a blood route is also available. This blood route may lead directly to the interstices of the nervous tissues. Flexner and Amoss<sup>9</sup> ascertained that an aseptic meningitis set up by an intraspinal injection of horse serum opened the way to the nervous tissues for the virus of poliomyelitis introduced into the blood, often leading to infection and paralysis which a blood injection alone does not accomplish. These authors<sup>10</sup> also report that after the subdural injection of horse serum, immunity principles passively introduced into the blood pass into the cerebrospinal fluid. These facts indicate that in endeavoring to influence the course of epidemic poliomyelitis in human beings, the following conditions should be observed: (1) early and prompt diagnosis and treatment; (2) intraspinal injection of immune serum; (3) injection of immune serum directly or indirectly into the blood. The coincident injection into the blood is indicated not only by the pathology of poliomyelitis but also by the physical phenomena involved, since it may be assumed that the tendency will be for an equilibrium to be established between the serum in the subarachnoid space and that in the blood. Increasing directly the serum in the blood should diminish the rapidity of outflow of that in the meninges. It happens fortunately that protein substances introduced into the low levels of the subarachnoid spaces pass into the blood more slowly than when introduced at higher levels.<sup>11</sup> Furthermore, it is possible to administer larger amounts of serum by the blood than intraspinally. Finally, the serum employed should be collected from cases which have recently passed through an attack of poliomyelitis, and not many years after, as has been the practice of Netter and others. It is to be supposed that the serum will contain a greater amount of immune substances in this early period than after the lapse of many years. A test carried out with a sample of blood serum taken from a patient on the 6th day of illness showed that it already contained the neutralizing principles.<sup>10</sup>

<sup>9</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

<sup>10</sup> Flexner and Amoss, *J. Exp. Med.*, 1917, xxv, 499.

<sup>11</sup> Dixon, W. E., and Halliburton, W. D., *J. Physiol.*, 1915-16, l, 198.

## CASE HISTORIES.

The serum employed in the series of cases to be reported was obtained from human beings who had recently recovered from an attack of epidemic poliomyelitis and who, at the time the serum was taken, were still paralyzed. All the donors of the serum except two had been convalescent for 8 weeks or less. The two exceptions had passed through the disease 2 years before.

The use of the serum from recently recovered persons, otherwise healthy, involves no risk of transferring the microorganism of poliomyelitis. In the first place, the virus has never been detected in the circulating blood of human beings even in the first days of the disease.<sup>12</sup> In the second place, even if minimal quantities of the virus are present in the circulating blood, the presence of the immunity principles in the serum would bring about its neutralization during the 48 hour period following the collection of the serum, during which sterility is being determined prior to its use.

The serum administered was collected under aseptic conditions, and was used uninactivated and unpreserved. The conditions surrounding the treatment of this series of cases were so carefully controlled that no risk was taken. On the other hand, one can now state that inactivation of the serum by heating to 53°C. for  $\frac{1}{2}$  hour or by the addition of 0.2 per cent tricresol does not impair the protective power.

*Mode of Administration.*—For the reasons given, we departed from the methods formerly employed and injected the serum not only intraspinaly but also into the blood. The quantity of serum which can safely be given to a child by intraspinal injection within a 12 hour period is obviously limited. In order to increase the quantity of immune bodies present in the fluids and tissues, even larger quantities were injected intravenously directly, or indirectly by way of the skin. The accompanying tables, although based on a relatively small number of cases, indicate the advantage of the larger volumes of serum administered. Some degree of selection was exercised in choosing cases for the treatment. The quantity of available serum was limited, and in view of the unproved value of the serum it was used in those

<sup>12</sup> Clark, P. F., Fraser, F. R., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 223.



cases in which the fever had not lasted over 48 hours; in a few instances the serum was given to patients who were critically ill, although the febrile period was of much longer duration.

For purposes of analysis and comparison, the cases treated with serum may be divided into two groups. The first group comprises the cases in which some degree of paralysis existed before the serum was administered; the second group includes the cases in which at the beginning of the treatment no paralysis could be detected by physical examination. The cases in the second group, however, were instances of poliomyelitis, since the clinical histories, general findings, and changes in the cerebrospinal fluid left no doubt on that point.

The first group of cases should be further subdivided into two classes: of these one class was under observation long enough for us to determine that the pathologic process in the spinal cord was extending and involving other regions; in the other class treatment was begun immediately in the home and continued after admission to the hospital. No information was, therefore, available to indicate whether the progress of the disease had already become arrested or not.

*Group I. Cases Showing Paralysis at the Beginning of the Treatment.*

*Class I. Paralysis Extending.*

*Case 1.*—Male; age 33 years.

Sept. 2, 1916. Malaise, headache, and vomiting. Sept. 3. Unable to bear weight on legs. Sept. 4. Admitted to hospital. Legs completely paralyzed. No tendon reflexes. Cerebrospinal fluid shows 280 cells per c.mm. and increased globulin. Bladder, bowels, diaphragm, abdominal and thoracic walls, and upper extremities normal. Sept. 5. Bladder paralyzed; no abdominal movement during respiration; using accessory muscles of respiration; deltoids weak; cyanosis.

On Sept. 5, 10 cc. of serum were injected intraspinally and 20 cc. intravenously. The cyanosis gradually increased, and death resulted from respiratory paralysis 10 hours after the injection. No obvious influence on the course of the disease was exerted by the serum.

*Case 2.*—Female; age 10 years.

Sept. 22, 1916. Headache, nausea, fever, and abdominal pain. Sept. 23. Temperature 103°F. Pain in back and in right leg; drags right foot. Sept. 25. Admitted to hospital. Temperature 102.6°F.; pulse 112; respirations 30. Neck and back muscles stiff; flaccid paralysis of both legs; bladder functions. Sept. 26, 6 p.m. Left deltoid paralyzed; dyspneic; employs accessory muscles of respira-

tion; no respiratory movement of abdominal wall; bladder paralyzed. Cerebrospinal fluid contains 270 cells per c.mm.; globulin increased.

On Sept. 26, 20 cc. of serum were given intraspinally and 60 cc. subcutaneously. Sept. 27, 8 p.m. Temperature 99.8°F.; pulse 102; respirations 30. Abdominal muscles move with respiration. Sept. 28. Abdomen moves freely; left deltoid responds but not yet normally; bladder inactive. Sept. 30. Bladder acting; no change in leg condition.

The history of the acute stage of this case readily divides itself into two periods. The first (a) takes in the time up to and including the paralysis of the legs, followed by a brief interval when (b) the abdomen, diaphragm, shoulder, and bladder became paralyzed. It was during the second period that the serum was administered. Coincidentally with the administration there was no further progress, but rather a rapid recession of the recent paralysis; the older paralytic condition was not obviously affected. Whether the phenomena described are of the nature of cause and effect cannot certainly be determined.

*Case 3.—Male; age 3 years.*

Sept. 26, 1916. Fever and vomiting. Sept. 27. Difficulty in swallowing. Sept. 28. Admitted to hospital. Temperature 101°F.; pulse 112; respirations 38. Paralysis of muscles of deglutition. Sept. 29. Complete paralysis of right side of face. 12 m. Temperature 104.2°F.; pulse 138; respirations 62. Very ill. 2 p.m. Cerebrospinal fluid, 208 cells per c.mm.; globulin slightly increased.

On Sept. 29, 15 cc. of serum were administered intraspinally, 20 cc. intravenously, and 40 cc. subcutaneously. Sept. 30. Temperature 99°F.; pulse 102; respirations 68. Marked improvement in general condition but no change in the degree of paralysis of the face or muscles of deglutition. Before discharge the paralysis had almost disappeared.

Whether the prompt subsidence of the severe general symptoms in this case is to be ascribed to the serum treatment cannot be stated positively. The fact that no extension of the paralysis occurred in spite of the severe symptoms may or may not have been due to the treatment. But in this instance, as in Case 2, the paralysis was extending at the time the serum was given and no further extension occurred. It is obvious that in the first case of this series, no result was accomplished. It should be mentioned, however, that as compared with the more energetic treatment subsequently employed that case received a very small quantity of the serum, which was administered late in the course of the disease.



*Class II. Paralysis Present.*

*Case 4.*—Female; age 33 years.

Oct. 16, 1916. Chilly sensations; abdominal pain; difficulty in walking. Oct. 17. Stiffness of neck; legs weak. Oct. 18. Admitted to hospital. Temperature 103°F.; pulse 104; respirations 34. Neck and back stiff; weakness of muscles of abdomen and left leg. None of the muscle groups are completely paralyzed. Cerebrospinal fluid, 50 cells per c.mm.; globulin increased.

On Oct. 18, 20 cc. of serum were given intraspinally and 50 cc. intravenously. Oct. 19. Paralysis stationary. Oct. 21. Temperature 99.6°F. No extension of the paralysis.

The serum was administered in this case probably within 24 hours of the onset of definite paralysis, but whether or not it influenced the progress cannot be stated.

*Case 5.*—Male; age 2 years.

Sept. 13, 1916. Fever and headache. Sept. 18. After an apparently normal interval, vomiting occurred and the neck and back became stiff. Sept. 20. Weakness of legs noticed. Admitted to hospital. Temperature 98.2°F.; pulse 108; respirations 44. Neck and back stiff; face slightly asymmetrical; abdominal wall relaxed; no reflexes; partial paralysis of muscles of legs. Cerebrospinal fluid, 37 cells per c.mm.; globulin increased.

On Sept. 20, 5 cc. of serum were given intraspinally and 30 cc. subcutaneously. The paralysis did not extend, but there was no immediate improvement of the paralyzed muscles.

The treatment in this case was given probably at the end of the 4th day of illness at a time when the paralysis had already been arrested. Apparently nothing definite was accomplished. Case 6, a sister of this patient, should be used for comparison.

*Case 6.*—Female; age 4 years.

Sept. 19, 1916. Fever, headache, and constipation. Sept. 20. Temperature 101.4°F.; vomiting. Facial asymmetry. 11 p.m. Admitted to hospital. Temperature 102°F.; pulse 140; respirations 32. Neck and back stiff; slight left facial paralysis; weak hamstring and quadriceps muscles on both sides; knee jerks and Achilles tendon reflexes increased. Cerebrospinal fluid cloudy and contains 920 cells per c.mm.; globulin increased.

On Sept. 20, 10 cc. of serum were given intraspinally and 25 cc. subcutaneously. Sept. 22. No extension of paralysis. Sept. 23. Temperature normal.

In this case the serum was given within 30 hours of the onset of the symptoms and at a time when weakness of the muscles of the thighs

was appearing. The temperature became normal in about 60 hours and no extension of the paralysis ensued (Text-fig. 5). Within 2 weeks recovery of all the weak muscles was complete. The striking point in the case is the large number of cells in the cerebrospinal fluid when the serum was injected.

*Case 7.*—Female; age 2 years.

Sept. 12, 1916. Headache and fretfulness. Sept. 14. Admitted to hospital. Temperature 101.8°F.; pulse 124; respirations 28. Weakness of flexors and extensors of right hip and of gastrocnemius. Achilles tendon reflexes absent. Cerebrospinal fluid contains 99 cells per c.mm. and increased globulin.

On Sept. 14, 5 cc. of serum were injected intraspinally and 30 cc. subcutaneously. Sept. 15. Temperature normal; paralysis stationary. There was never any extension, but gradual improvement of the partially paralyzed muscles. The temperature curve is shown in Text-fig. 1.

*Case 8.*—Male; age 22 months.

Sept. 9, 1916. Child ill. Sept. 10. Admitted to hospital. Temperature 102.6°F.; pulse 136; respirations 36. Weakness of left quadriceps anterior.

On Sept. 10, 5 cc. of serum were administered intraspinally at home before removal to the hospital. Sept. 11. Left thigh muscles weaker. 20 cc. of serum given subcutaneously. Sept. 13. Temperature normal. Partial paralysis unchanged.

*Case 9.*—Female; age 3 years.

Oct. 12, 1916. Child feverish and irritable. Oct. 14. Left leg partially paralyzed. Oct. 15. Admitted to hospital. Temperature 99.8°F.; pulse 120; respirations 32. There is stiffness of the neck and back, weakness of the extensors of the left foot, and toe-drop. Cerebrospinal fluid contains 115 cells per c.mm.; globulin increased.

On Oct. 15, 15 cc. of serum were injected intraspinally and 40 cc. subcutaneously. The temperature fluctuated irregularly between 98.6° and 100° F. for 2 weeks. The weakness of the foot and leg increased somewhat. 1 month later some degree of impairment was present in the left quadriceps, hamstrings, peroneals, and toe extensors, while the anterior tibial group was completely paralyzed.

The serum was injected in this case within about 72 hours of the first appearance of any symptoms and at a time when only slight and limited paralysis existed. Nevertheless, the muscular impairment progressed slowly in the muscles first affected and into neighboring muscles, but the degree of paralysis never became severe.

*Case 10.*—Male; age 15 months.

Sept. 9, 1916. Child feverish and sweating; vomited. Admitted on same day. Temperature 99.6°F.; pulse 132; respirations 36. The neck and back are stiff

and there is doubtful weakness of the right deltoid. Cerebrospinal fluid shows 30 cells per c.mm.; globulin increased.

On Sept. 9, 5 cc. of serum were given intraspinally. There was no further involvement of the right deltoid and the temperature remained normal during the stay in the hospital.

This child was seen within a few hours of the onset of the first symptoms and the serum injection was given at the home before removal to the hospital. No paralysis developed.

*Case 11.*—Female; age 2 years.

Sept. 27, 1916. At noon the child was feverish. 10 p.m. Stiffness of the neck and back, weakness of the left quadriceps and peroneal muscles, and slight weakness of the intercostals. The patient, whose brother was already in the hospital, was admitted at once. Temperature 101°F.; pulse 110; respirations 32. Cerebrospinal fluid, 30 cells per c.mm.; globulin increased.

On Sept. 27, 10 cc. of serum were given intraspinally and 30 cc. subcutaneously. Sept. 28. After 24 hours the temperature was 100°F.; no extension of muscular weakness. Sept. 29. Temperature normal. Muscular impairment gone. No abnormality on discharge.

The serum was administered to this child within 12 hours of the first appearance of the symptoms, and although a degree of weakness of certain muscles was already present, it quickly disappeared.

*Case 12.*—Male; age 3½ years.

Sept. 12, 1916. Headache and fever. Sept. 13. Temperature 103.8°F. Stiffness of neck and back; weakness of muscles of right ankle; toe-drop. Cerebrospinal fluid, 250 cells per c.mm.; globulin increased.

On Sept. 13, 10 cc. of serum were injected subdurally and 40 cc. subcutaneously. Sept. 14. Temperature 99.6°F. Weakness of muscles diminished rapidly.

The serum was administered within 24 hours of the onset of symptoms and at a time when several muscle groups were impaired. The temperature quickly fell to normal and the muscular weakness soon disappeared.

In considering Cases 1 to 12 the points that can be made definitely are: (1) the diagnosis was clearly established in each; (2) there was some grade of paralysis present in each; and (3) with the exception of Cases 1, 8, and 9, there was either no increase of the existing muscular weakness or paralysis, or there was prompt improvement in these conditions.

*Group II. No Paralysis at the Time of Treatment.*

In the following series of fourteen cases, no paralysis was detected up to the time that the serum was administered. One reservation should, however, be made: in the early stages of the disease, some of the patients were too ill to warrant a full examination of all the muscle groups and hence a degree of weakness or paralysis may have sometimes been overlooked. Moreover, the quantity of serum administered varied considerably, depending upon circumstances. The cases will be considered in the order of the amounts of serum given.

*Case 13.*—Male; age 8 years.

Sept. 19, 1916. Headache, fever, and drowsiness. Sept. 20. Same condition; neck and back stiff. Sept. 21. Admitted to hospital. No paralysis detected. Temperature 102.5°F.; pulse 102; respirations 32. Cerebrospinal fluid, 850 cells per c.mm.; globulin increased.

On Sept. 21, 5 cc. of serum were given intraspinally and 15 cc. subcutaneously. Sept. 24. Temperature normal. In the interim weakness of the following muscle groups developed: deltoids, pectorales major, rotators (outward) of arms, flexors of hip, quadriceps and hamstrings, and abdominal wall.

The serum was administered within 48 hours of the onset of symptoms. The amount given intraspinally was small. Weakness subsequently developed in many muscle groups, but in none was the paralysis complete.

*Case 14.*—Male; age 19 months.

Sept. 19, 1916. Vomiting, attributed to improper food. Sept. 20. Fever; neck and back stiff; muscular twitchings. Admitted to hospital. Temperature 103°F.; pulse 124; respirations 32. While there was muscular stiffness, no weakness was detected. Cerebrospinal fluid, 120 cells per c.mm.

On Sept. 20, 5 cc. of serum were injected intraspinally and 20 cc. subcutaneously. The fever persisted, and paralysis developed, involving ultimately the muscles of respiration. Sept. 23. Died.

Obviously no influence on the course of the disease was exerted by the serum. The experience gained later now indicates that the injection should have been repeated.

*Case 15.*—Female; age 4 years.

Sept. 23, 1916. Fever and headache; irritable. Sept. 24. Neck and back stiff; muscular twitching. Admitted to hospital. Temperature 103.6°F.; pulse

128; respirations 34. No muscular impairment detected. Cerebrospinal fluid, 360 cells per c.mm.; globulin increased.

On Sept. 24, 10 cc. of serum were given intraspinally and 20 cc. subcutaneously. Sept. 25. Flaccid paralysis of right arm and shoulder girdle with rapid extension and involvement of muscles of respiration. Died.

Autopsy showed severe poliomyelitic lesions of the cervical cord and medulla.

Obviously this case was not benefited by the serum injection, since paralysis developed a few hours after the treatment and extended rapidly.

*Case 16.*—Female; age 8 years.

Sept. 23, 1916. Headache; restless. Sept. 24. Temperature 102°F. Neck stiff. Cerebrospinal fluid, 730 cells per c.mm.; globulin increased.

Within 6 hours of the onset 10 cc. of serum were administered intraspinally and 20 cc. subcutaneously. The temperature fell slowly and reached normal on the 4th day. During this period weakness of the muscles of both arms and possibly slight weakness of the left leg appeared; complete paralysis was never present. Improvement was rapid, and almost complete recovery of lost strength was made in a short time.

*Case 17.*—Female; age 2 years.

Sept. 17, 1916. Fever; drowsy; muscular twitching; convulsions. Sept. 19. Admitted to hospital. Cerebrospinal fluid, 60 cells per c.mm.; globulin increased. Temperature 104.2°F.; pulse 132; respirations 32. Neck and back stiff; no muscular weakness.

On Sept. 19, 6 cc. of serum were given intraspinally and 25 cc. subcutaneously. Within 24 hours the temperature was normal. No muscular weakness developed.

*Case 18.*—Male; age 5 years.

Sept. 14, 1916. Temperature 103°F.; headache; drowsy. Sept. 15. Neck and back stiff. Temperature 104°F. Admitted to hospital. Temperature 103.4°F.; pulse 126; respirations 50. No weakness of muscles. Cerebrospinal fluid, 379 cells per c.mm.; globulin increased.

Within 24 hours of the onset 5 cc. of serum were given intraspinally and 25 cc. subcutaneously. Sept. 16. Temperature 101°F. 5 cc. of serum were introduced intraspinally. Cerebrospinal fluid, 249 cells per c.mm. Sept. 17. Temperature normal. No muscular weakness ever developed.

*Case 19.*—Male; age 4 years.

Sept. 17, 1916. Temperature 102°F.; pain in back. Sept. 18. Temperature 102.4°F. Neck and back stiff. Cerebrospinal fluid, 150 cells per c.mm.; increased globulin. Admitted within 24 hours of onset.

On Sept. 18, 10 cc. of serum were injected intraspinally and 25 cc. subcutaneously. 15 hours later the temperature became normal. No muscular weakness developed.



*Case 20.*—Male; age 13 months. Cousin of Case 19. .

Sept. 17, 1916. Drowsiness and fever. Sept. 18. Cerebrospinal fluid clear and contains 75 cells per c.mm.; globulin increased. The patient was given 10 cc. of serum subdurally and was shortly afterwards brought to the hospital. On admission, temperature 101.4°F.; pulse 120; respirations 26.

Immediately after admission a subcutaneous injection of 25 cc. of serum was given. The temperature rose to 104°F. and fell to normal 22 hours afterwards. There was no subsequent weakness or paralysis.

This patient was treated about 18 hours after the first symptoms. The temperature previous to treatment is not known. After a rise to 104°F. the temperature fell rapidly, reaching normal about 22 hours after the first injection (Text-fig. 2). The child developed no subsequent weakness or paralysis.

*Case 21.*—Female; age 3½ years.

Sept. 3, 1916. Headache; temperature 102°F. Sept. 4. Temperature 104°F. Sept. 5. Temperature 101.5°F. Stiffness of neck. Sept. 6. Cerebrospinal fluid clear and contains 230 cells per c.mm.; globulin increased. Admitted to hospital the same afternoon. On admission, temperature 102.4°F.; pulse 130; respirations 42. Stiffness of neck and back; no weakness or paralysis noted.

Immediately after admission 5 cc. of serum were injected subdurally and 40 cc. subcutaneously. Temperature reached normal 32 hours after treatment. No definite weakness or paralysis developed subsequently.

In this case treatment was begun about 3 days after the initial symptoms and at a time when paralysis had not appeared. Treatment was followed by a rapid drop in temperature and no weakness or paralysis developed during the period of observation.

*Case 22.*—Female; age 3½ years.

Oct. 7, 1916. Fatigue, irritability, and vomiting. Oct. 8. Fever and a mild delirium. Oct. 10. Fever persisted; drowsiness. Admitted to hospital. On admission, temperature 101.6°F.; pulse 132; respirations 28. Stiffness of neck and back. Clear cerebrospinal fluid with 88 cells per c.mm.

On Oct. 10, 20 cc. of serum were injected subdurally and 30 cc. subcutaneously. Temperature rose to 104°F. that night and there was slight nystagmus. Lumbar puncture yielded a slightly turbid fluid under considerable pressure. About 30 cc. of fluid were withdrawn from the spinal canal. On the following morning the nystagmus had disappeared and 36 hours after treatment the temperature reached normal. Cultures of the turbid fluid were sterile. Turbidity was due to the presence of a large number of polymorphonuclear leukocytes. No weakness or paralysis developed subsequent to treatment.



This patient was treated 3 days after the initial symptoms when there was no evidence of weakness or paralysis. Following the treatment there was evidence of increased irritation of the meninges and increased pressure in the subdural space which was relieved by lumbar puncture and withdrawal of fluid. The temperature reached normal 36 hours after the injection and the child did not develop any weakness or paralysis subsequently. The serum used in this case contained considerable fat; whether or not this played a part in the reaction following the injection cannot be stated definitely. This was the only instance in which any such reaction was obtained, although other cases were treated with portions of the same serum.

*Case 23.*—Male; age 5 years.

Oct. 3, 1916. Vomiting. Oct. 4. Fever; vomiting persisted. Clear cerebrospinal fluid with 250 cells per c.mm. Admitted to hospital. On admission, temperature 101°F.; pulse 130; respirations 30. Stiffness of neck and back; no weakness or paralysis.

15 cc. of serum were immediately given subdurally and 40 cc. subcutaneously. Temperature rose to 103°F. and fell to normal 12 hours later. There was no subsequent weakness or paralysis.

This patient was treated about 30 hours after what seemed to be the initial symptoms. At the time of treatment there was no demonstrable involvement of any muscle group. The temperature dropped to normal 12 hours after treatment and the child did not show any weakness or paralysis at any time.

*Case 24.*—Female; age 1½ years.

Oct. 11, 1916. Temperature 101°F. Oct. 12. Stiffness of the neck and back with muscle tenderness. Temperature 103°F. No demonstrable weakness. Admitted to hospital. On admission, temperature 103°F.; pulse 118; respirations 28. Clear cerebrospinal fluid with 40 cells per c.mm.; globulin increased.

On Oct. 12, 25 cc. of serum were injected subdurally and 35 cc. subcutaneously. Temperature dropped to normal within 16 hours after treatment. At no time was there any demonstrable weakness present.

This patient was treated about 30 hours after the mother first noticed fever, and at a time when there was no demonstrable weakness of any muscles. The temperature became normal in less than 24 hours after treatment (Text-fig. 3). An older sister of this patient was admitted to the hospital on the day previous to the one on which

this case was admitted. The sister had been sick for a period of 4 days with temperature as high as 104°F. She was admitted at the end of the febrile period, 4 days after the onset, and at the time of admission lumbar puncture showed the presence of a clear fluid with 76 cells and increased globulin. She received no serum and developed no weakness or paralysis.

*Case 25.*—Male; age 5 years.

Oct. 5, 1916. Irritability and fever. Oct. 6. Fever persisted; constipation. Oct. 7. Admitted to hospital. On admission, temperature 103°F.; pulse 104; respirations 28. Marked stiffness of neck and back; active reflexes; positive Kernig. No demonstrable weakness of any muscle group. Clear cerebrospinal fluid with 260 cells per c.mm.; globulin increased.

On Oct. 7, 22 cc. of serum were given subdurally and 45 cc. subcutaneously. The temperature fell to normal within 22 hours after treatment and the patient did not develop any weakness or paralysis.

This patient was treated about 48 hours after onset of the first symptoms and within 22 hours after treatment the temperature was normal (Text-fig. 4). No weakness or paralysis developed. A younger sister of this patient was admitted to the hospital 2 days previously with flaccid paralysis of both lower extremities. In this case onset had occurred 5 days before admission and the temperature had fallen to normal and paralysis had already set in when she was admitted.

*Case 26.*—Male; age 23 years.

Dec. 9, 1916. Vomiting and headache. Stiffness of neck, hyperesthesia, and asymmetry of patellar reflexes. Kernig's sign present on right side. Temperature 101°F. No demonstrable weakness of any muscle group. Clear cerebrospinal fluid with 40 cells per c.mm.; globulin increased.

38 hours after onset the patient received 20 cc. of serum subdurally and 100 cc. intravenously. Following treatment the temperature fell to normal within 48 hours, the hyperesthesia disappeared within that time, and no paralysis or weakness developed.

This patient, an adult, was treated within 38 hours after the initial symptoms appeared, at a time when there was no demonstrable involvement of the muscles. The temperature rapidly fell to normal, and no weakness or paralysis developed subsequently.

The more important facts relating to the twenty-six cases described are collected in Table I.

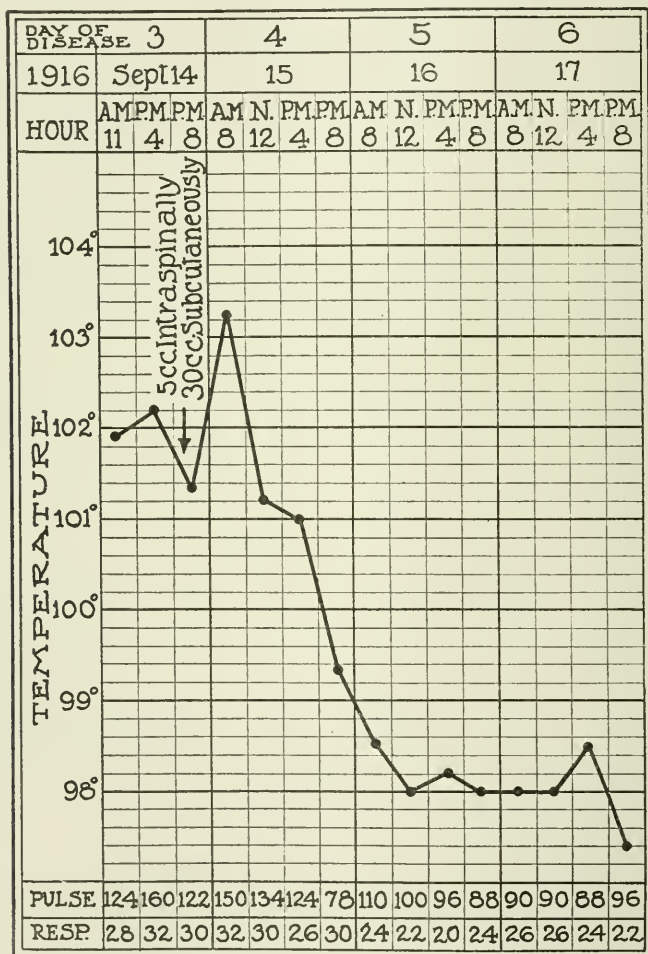
TABLE I.  
Summary of Cases That Received Serum Treatment.

Case No.	Age.	Clinical condition.	Spinal fluid.		Time between onset of disease and treatment.	Serum administration.			Temperature before treatment.	Time between treatment and normal temperature.	Total amount of serum given.	Result.
			No. of cells.	Globulin.		Subdural.	Subcutaneous.	Intravenous.				
1	33 yrs.	Ascending paralysis of legs, bladder, and abdominal and thoracic muscles.	280	++	72	10		20	97° 101.2	hrs.	30	Died 10 hrs. after treatment.
2	10	Ascending paralysis of legs, bladder, abdominal wall, diaphragm, left deltoid, and right facial muscles.	270	++	96	20	60		101.4	24	80	Deltoid and diaphragm functioning 24 hrs. later. Voided 4 days later.
3	3	Right facial and deglutition muscles paralyzed.	208	+	72	15	40	20	104.2	18	75	No further progress of paralysis. Subsequent improvement marked.
4	33	Weakness of left leg and abdominal muscles.	50	+	54	20		50	103.0	60	70	No progress of paralysis. Subsequent improvement.
5	2	Weakness of sternocleidomastoids, both legs, and intercostals.	37	+	96	5	30		98.2		35	No essential change.
6	4	Partial paralysis.	920	++	30	10	25		102.0	60	35	" increase. Complete recovery rapid.
7	2	Weakness of flexors of right thigh.	99	+	48	5	30		101.4	24	35	No progress of lesion. Subsequent improvement.
8	1½	Weakness of left quadriceps.			48	5	20		102.0	48	25	Slight increase in weakness.

9	3	Weakness of left anterior tibialis.	115	+	72	15	40	100.2	Low fever for 2 wks.	55	Definite increase in extent of paralysis of left leg.
10	1 $\frac{3}{4}$	Weakness of right deltoid.	30	++	24	5		101.0	24	5	No increase in weakness.
11	2	Weakness of left peroneal and quadriceps and slight weakness of intercostals.	30	=	12	10	30	101.0	30	40	" " " paralysis. Rapid disappearance of weakness noted on admission.
12	3 $\frac{1}{2}$	Slight right toe-drop.	250	++	24	10	40	103.0	24	50	No paralysis.
13	8	No paralysis. Left knee jerk diminished.	850	++	48	5	15	102.5	60	20	Developed partial paralysis in arms, legs, and abdominal muscles.
14	1 $\frac{7}{8}$	No paralysis.	120		31	5	20	103.0		25	Died in 66 hrs.
15	4	"	360	++	36	10	20	103.2		30	" " 28 "
16	8	"	730	++	6	10	20	102.0	96	30	Slight partial paralysis of both arms. Subsequent recovery complete.
17	2	"	60	=	36	6	25	102.0	22	31	Subsequent rise to 102°F. Variable temperature for 5 days. No paralysis.
18	5	"	379	+	22 $\frac{1}{2}$	10 (2 doses).	25	103.4	32	35	No paralysis.
19	4	"	150	++	24	10	25	102.4	15	35	" "
20	1 $\frac{1}{2}$	"	75	++	18	10	25	101.4	22	35	" "
21	3 $\frac{1}{2}$	"	230	++	66	5	40	102.4	32	45	" "
22	3 $\frac{1}{2}$	"	88		76	20	30	101.6	36	50	" "
23	5	"	250		30	15	40	101.2	12	55	" "
24	1 $\frac{1}{2}$	"	40	+	31	25	35	103.0	16	60	" "
25	5	"	260	+	48	22	45	103.2	22	67	" "
26	23	"	40	++	38	20		101.0	48	120	" " Hyperesthesia disappeared in 48 hrs.

## DISCUSSION.

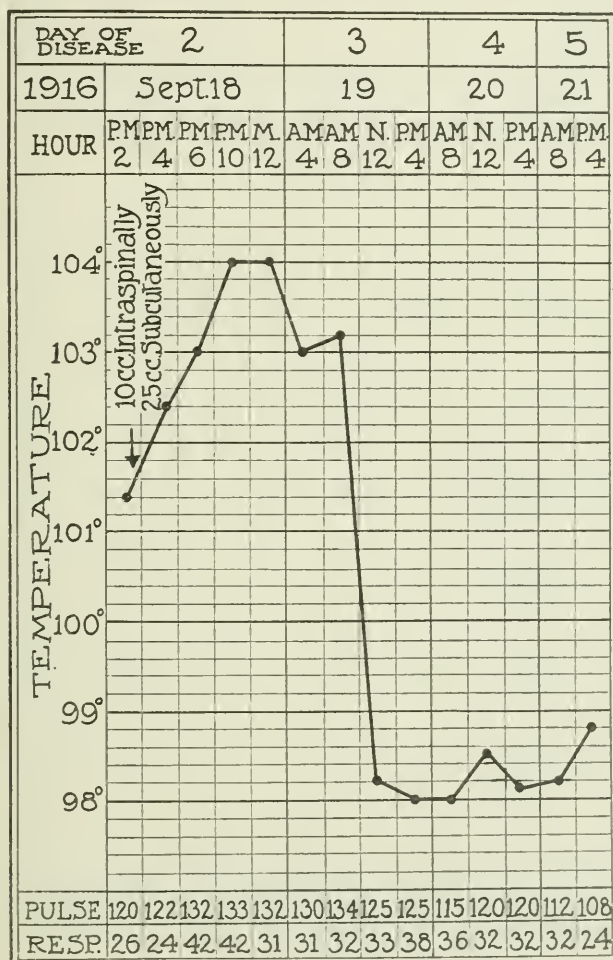
Since the data in the twelve cases which showed paralysis at the time serum was first given are less complete, and the control of the conditions was less perfect, we shall dismiss them with few comments.



TEXT-FIG. 1. Temperature chart of Case 7.

One patient died 10 hours after the serum was given, two patients suffered some degree of extension of the paralysis, while the remaining nine showed no extension of the paralysis.

The circumstances surrounding the group of fourteen cases in which no paralysis was detected at the time serum was administered are more favorable for a conclusion. Two of the patients of this group developed respiratory paralysis and died; and two others de-



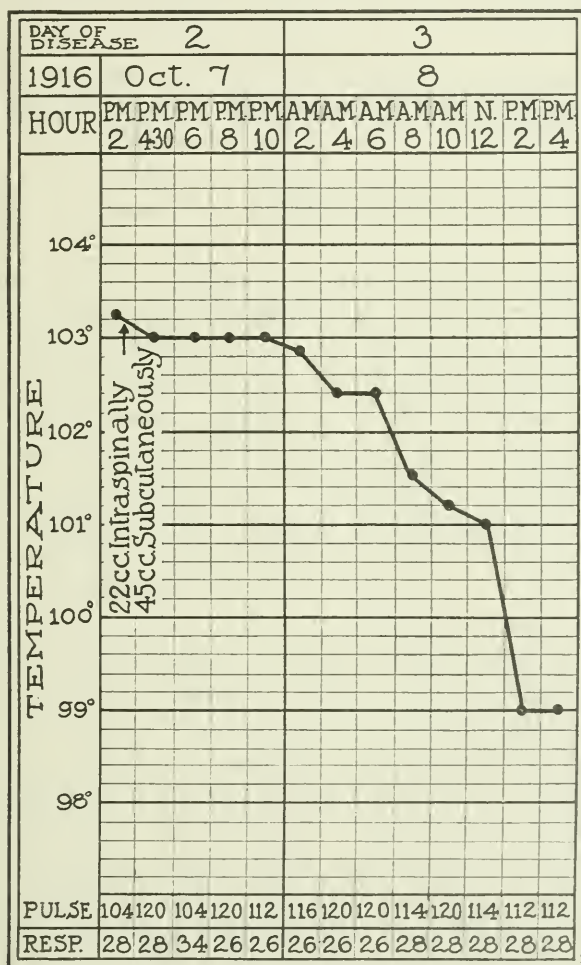
TEXT-FIG. 2. Temperature chart of Case 20.

veloped some degree of weakness or partial paralysis of certain muscle groups. The ten remaining cases (71 per cent) never showed any detectable weakness.





first is the following sharp rise in temperature, of brief duration; the second is the increase in stiffness of the neck and even the accentuation of the opisthotonos already present. The former has been



TEXT-FIG. 4. Temperature chart of Case 25.

frequently noted especially after the intraspinal injection of salvarsanized serum employed in the treatment of syphilis of the nervous system;<sup>13</sup> the latter results from the irritation or even transient aseptic

<sup>13</sup> Ellis, A. W. M., and Swift, H. F., *J. Exp. Med.*, 1913, xviii, 428.

tic inflammation incited in the meninges by the serum. Coincident with this condition the cerebrospinal fluid may become turbid (Case 22) from emigrated leukocytes; but the inflammation lasts only a day or two and is in no way a source of danger.

In each case there was an increase in the number of cells in the fluid withdrawn by lumbar puncture as well as excess of globulin. In several instances the cell count was high (Cases 6, 13, and 16). In nine of the fourteen included in the second group, the count exceeded 100 cells per c.mm. As far as this is an index in pointing to the probable development of paralysis, it is favorable to the serum treatment.

This moderate number of cases is sufficient to demonstrate the harmlessness of the serum when introduced intraspinally under suitable conditions. The gravity method of injection was always employed. Each sample of serum was tested bacteriologically and found to be sterile; and in every instance a Wassermann test was employed to exclude syphilitic taint. Particular care was taken to obtain serum free from corpuscles or hemoglobin. Only perfectly clear and colorless samples were employed for injection. We believe that attention should be directed to these points in order that the reaction to the presence of the serum, a foreign body in the subarachnoid space, may be reduced to the minimum.

Finally, we had in mind that in acute poliomyelitis to a greater extent than in epidemic meningitis, the substance of the spinal cord and medulla suffer injury. On that account they may suffer even more readily the ill effects of increased intracranial pressure. In poliomyelitis the lumen of the blood vessels is already encroached upon by the perivascular infiltration. Hence the serum should be injected under low pressure and only after the previous withdrawal of a greater amount of spinal fluid than the serum injected. Should symptoms referable to increased intracranial pressure arise, they may be relieved by prompt withdrawal of the injected fluid. In one instance only (Case 22) in our series did we resort to the withdrawal as a precautionary measure, although the removal was not urgently demanded.

In estimating the probable effects of the serum other considerations

arise. The first relates to the period of the disease at which the serum is injected. If there is analogy between this disease and epidemic meningitis, for example, then the earlier the serum is injected, the more pronounced should be the effects. In other words, it should theoretically be easier to prevent paralysis altogether or to limit its extent and intensity than to bring about its rapid retrogression. This fact follows from the pathology of poliomyelitis<sup>14</sup> and from the manner in which automatic arrest of the pathologic process takes place.

The treatment was begun with definite plans for administering the serum early in the disease and in large amounts. The time limit between onset and treatment was set at 48 hours. It is obviously impossible to adhere strictly to this rule, but out of the twenty-six cases reported, eighteen were treated within this time. Apparently the best results are obtained in cases treated within 30 hours after onset, though beneficial results were obtained in one instance as late as 96 hours after onset (Table II).

The serum administered was obtained from persons recently recovered from poliomyelitis, at which period it is supposed to contain immune substances in greatest concentration. We suggest that if serum is used from patients whose attacks are more remote, correspondingly larger doses should be employed.

Recovery from poliomyelitis depends upon a process of self-immunization, and the indications are that the immunization takes place rapidly. On the 6th day of the disease, neutralizing immune bodies are already present in readily detectable quantities in the blood.<sup>10</sup> Therefore, what the therapeutic employment of the serum seeks to accomplish is the anticipation by artificial means of this process of autoimmunization. Probably also the artificial may have advantages over the automatic method. The latter operates chiefly by the medium of the blood, although doubtless the increased permeability of the meninges and choroid plexus in the damaged nervous organs permits an escape of immune bodies into the cerebrospinal fluid. It has been shown by direct experiment that the neutralizing immune bodies pass into the aseptically inflamed cerebrospinal fluid.<sup>10</sup> But

<sup>14</sup> Flexner and Lewis, *J. Exp. Med.*, 1910, xii, 227.

TABLE II.

*Effect of Early Administration of Serum.*

Case No.	Time between onset and treatment.	Result.	
	<i>hrs.</i>		
2	96	Marked improvement.	+
5	96	No change.	0
22	76	" paralysis.	+
3	72	Marked improvement.	+
9	72	Increase in paralysis.	-
1	72	Died in 10 hrs.	-
21	66	No paralysis.	+
4	54	Improvement.	+
7	48	No increase in paralysis.	+
8	48	Slight " " "	-
13	48	" " " "	-
25	48	No paralysis.	+
26	38	" "	+
15	36	Died in 28 hrs.	-
17	36	No paralysis.	+
14	31	Died in 66 hrs.	-
24	31	No paralysis.	+
6	30	" "	+
23	30	" "	+
19	24	" "	+
12	24	" "	+
10	24	" "	0
18	22½	" "	+
20	18	" "	+
11	12	Improvement.	+
16	6	Slight increase in paralysis.	-

\* In the tables + indicates improvement in the condition of the patient; 0 indicates no change; and - indicates that the disease was not checked.

it is generally conceded that the most direct route to the interstices of the central nervous organs is by way of the cerebrospinal fluid.<sup>15</sup> Hence the introduction of the immune serum intraspinally and intravenously at the same time should afford the quickest and most effective way of supplying the nervous tissues with the immunity substances which neutralize and inactivate the virus of poliomyelitis.

It has been stated, moreover, that larger amounts of serum should

<sup>15</sup> Flexner, S., *J. Am. Med. Assn.*, 1913, lxi, 447, 1872.

be administered in the early stages, in which the greatest benefit is expected. Table III shows that the best results are obtained in cases receiving a total of more than 30 cc. of serum.

TABLE III.  
*Effect of the Amount of Serum Administered.*

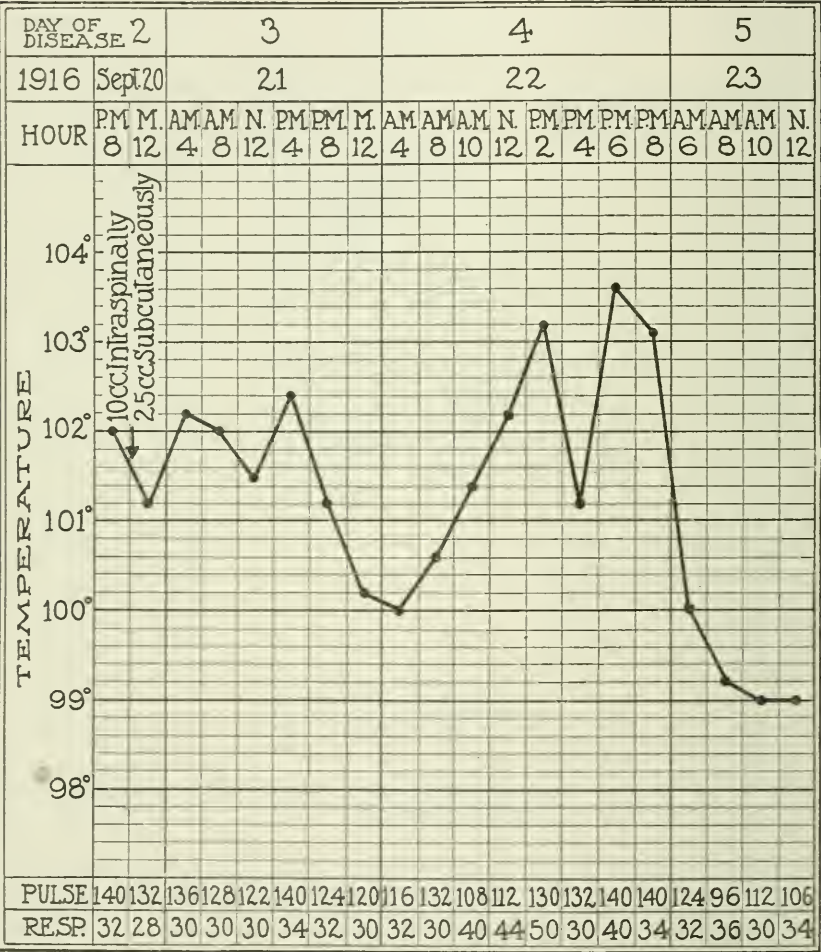
Case No.	Total amount of serum given.	Result.	
	cc.		
10	5	No increase in weakness.	0
13	20	Slight " " paralysis.	—
8	25	" " " "	—
14	25	Died in 66 hrs.	—
1	30	" " 10 "	—
15	30	" " 28 "	—
16	30	Slight increase in paralysis.	—
17	31	No paralysis.	+
5	35	" change.	0
7	35	" increase in paralysis.	0
18	35	" paralysis.	+
19	35	" "	+
20	35	" "	+
6	35	" "	+
11	40	" "	+
21	45	" "	+
12	50	" "	+
22	50	" "	+
9	55	Definite increase in paralysis.	—*
23	55	No paralysis.	+
24	60	" "	+
25	67	" "	+
4	70	Improvement.	+
3	75	Marked improvement.	+
2	80	" "	+
26	120	No paralysis.	+

\* Serum given 72 hours after onset of symptoms.

The patients who received a total of more than 30 cc. within 48 hours after the onset, without regard to the clinical condition, age, or temperature, seemed to be benefited in every case but one, and in this case the temperature quickly dropped to normal (Table IV).



It seems highly desirable, therefore, to administer at least 50 cc. of serum in every case and if possible to treat cases within 30 hours after the onset of the first symptoms.



TEXT-FIG. 5. Temperature chart of Case 6.

The plan of giving single large doses was strictly adhered to in this series, but a study of the temperature charts and clinical notes indicates that in some cases either larger doses or a second dose of serum

TABLE IV.

*Results in Cases Treated within 48 Hours after Onset with More than 30 Cc. of Serum.*

Case No.	Time between apparent onset and treatment.	Total amount of serum given.	Result.
	<i>hrs.</i>	<i>cc.</i>	
7	48	35	0
25	48	67	+
26	38	120	+
17	36	31	+
24	31	60	+
6	30	35	+
23	30	55	+
19	24	35	+
12	24	50	+
18	22½	35	+
20	18	35	+
11	12	40	+

should have been given. Case 6 received in all 35 cc. of serum and recovered rapidly without paralysis. However, the temperature chart suggests that a second dose of serum should have been given on the 4th day of the disease (Text-fig. 5).

## CONCLUSIONS.

1. Serum taken from recently recovered cases of poliomyelitis may be employed in its treatment and probably yields the best results.
2. When sterile for ordinary bacteria, free of corpuscles and hemoglobin, and when injected by the gravity method, observing well known rules of caution, it may be employed without danger.
3. The serum should be injected both intraspinally and intravenously, the latter either directly or by way of the subcutaneous tissues.
4. The earlier in the course of the disease the serum is employed in suitable doses, the more promise there is of benefit.
5. The action of the serum appears to be more precise and definite in arresting paralysis than in rapidly bringing about its retrogression.
6. The decision to employ the serum should rest upon a clinical examination supported by the results of the microscopic and chemical study of the cerebrospinal fluid.
7. The question of multiple and repeated injections of the serum

has not yet been worked out. In the cases here reported and especially in the group in which no paralysis existed at the time of the first injection, the pathologic process either did not progress at all, or where there was extension, as in Cases 14 and 15, the upper segment of the spinal cord became rapidly involved, and was followed by respiratory paralysis and death. Probably in cases in which some degree of muscular weakness develops soon after the injection of serum, reinjection 12 to 24 hours later may be advantageous. The temperature curve may serve to indicate the time for reinjection.

8. The favorable results thus far achieved in human beings by means of the immune serum support and extend those obtained experimentally in monkeys and indicate, as was foreseen, that the milder or less fatal form of poliomyelitis appearing in man is even more amenable to the serum treatment than is the highly fatal disease produced by inoculation in monkeys.

## EXPERIMENTS ON THE RÔLE OF LYMPHOID TISSUE IN THE RESISTANCE TO EXPERIMENTAL TUBER- CULOSIS IN MICE.

### II. EFFECT OF CANCER IMMUNITY ON RESISTANCE TO TUBERCULOSIS.

BY HERBERT D. TAYLOR, M.D., AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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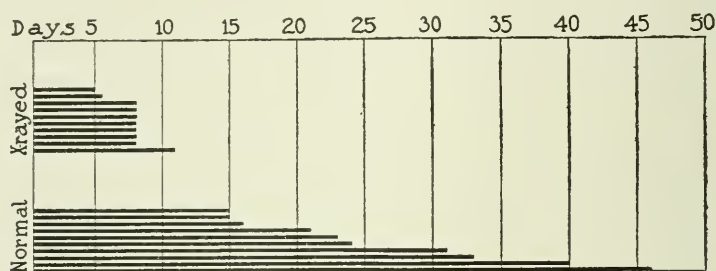
An investigation recently reported from this laboratory seems to bear out the conception that the lymphocyte plays a part in the resistance of the animal to tuberculous infection.<sup>1</sup> This work had for its starting-point the observation of Lewis and Margot<sup>2</sup> that mice experimentally infected with bovine tubercle bacilli developed splenic enlargement. Lewis and Margot also showed that animals splenectomized about 3 weeks before the injection of the tubercle bacilli exhibited greater resistance to the infection than did intact animals. In a study in this laboratory of blood changes after splenectomy, it was observed that the majority of mice so treated developed a marked lymphocytosis by the 19th to the 21st day after the operation.<sup>3</sup> It was thought probable, therefore, that this lymphocytosis might be a factor in causing the greater resistance displayed by the splenectomized animals. The following experiments confirm this view. Mice splenectomized and then exposed to repeated small doses of x-ray, which had been demonstrated to affect primarily the lymphoid

<sup>1</sup> Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

<sup>2</sup> Lewis, P. A., and Margot, A. G., *J. Exp. Med.*, 1914, xix, 187.

<sup>3</sup> L. B. Lange and Jas. B. Murphy, in an unpublished study of the blood of fifty mice before, and at intervals after splenectomy, showed that immediately after the operation there was a fall in the total lymphoid count. But by the 19th to the 21st day, 75 per cent of the animals counted showed an average increase of 11,000 lymphocytes per c.mm., or a gain of over 100 per cent above their normal count. The 25 per cent which did not gain averaged a loss of about 8 per cent of their former count.

organs,<sup>4</sup> proved to be more susceptible instead of more resistant to infection than were either normal animals or animals splenectomized alone (Text-fig. 1). Intact x-rayed mice were likewise highly susceptible to infection with the bovine tubercle bacilli. Morton<sup>5</sup> has observed also that the x-rayed guinea pig is more susceptible to infection with the human type of the tubercle bacillus than is the normal animal.



TEXT-FIG. 1. Each horizontal line represents the time of survival of a mouse after infection with bovine tubercle bacilli. The first group received a series of small doses of x-ray before inoculation, and the average duration of life was 7.8 days. The second group was normal and averaged 26.4 days of life after inoculation.

### *The Use of Cancer Immunity as a Method for Producing Lymphocytosis.*

As this evidence is of an indirect nature, it seemed desirable to obtain more direct data bearing on this conception. The present communication deals with the influence of a pronounced lymphocytosis induced by other means than splenectomy on the resistance of the mouse to tuberculous infection. We have already shown that mice immunized against and then inoculated with one of the transplantable mouse cancers developed a marked lymphoid reaction in the blood which lasted several weeks.<sup>6</sup> This method, therefore, affords a convenient way of obtaining what may be regarded as non-specific high blood lymphocytic reaction which lasts long enough to answer the experimental requirement.

<sup>4</sup> Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

<sup>5</sup> Morton, J. J., *J. Exp. Med.*, 1916, xxiv, 419.

<sup>6</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.



*Experiment 1.*—Twenty mice were injected subcutaneously with 0.3 cc. of defibrinated mouse blood. These, with twenty control animals, were inoculated 10 days later with a transplantable mouse carcinoma. At the end of 3 weeks 85 per cent of the control mice had developed tumors, while only one of the surviving sixteen immunized animals showed tumor. The fifteen immune animals, with ten normal mice from the same lot were each injected intraperitoneally with 3 mg. of a 9 day culture of bovine tubercle bacilli from a glycerol agar slant, taken up in 0.5 cc. of normal salt solution. The mice were placed in individual jars in order to prevent loss from epidemic disease. Autopsies were performed as soon after death as possible, and films were taken from the peritoneal fluid, kidneys, liver, spleen, lungs, and heart's blood. The gross and micropathology will not be described at this time. The distribution of the microorganisms is shown in Table I. The average survival after inoculation for the control animals was 20.3 days, while that of the cancer immune mice was 47.7 days; that is, they lived more than twice as long. The rate at which the mice died is shown in Text-fig. 2.

TABLE I.

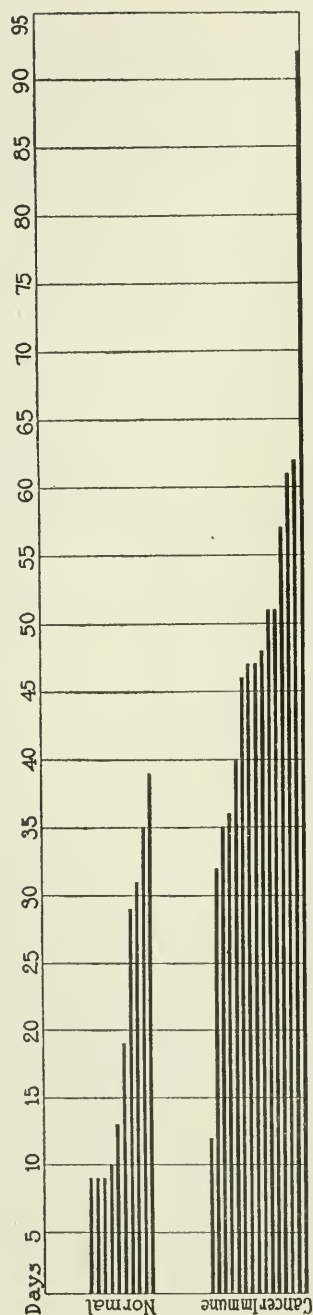
*Distribution of Tubercle Bacilli in the Cancer Immune Mice and the Control Mice from Experiment 1.*

Group.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidneys.
	days	per cent	per cent	per cent	per cent	per cent	per cent
Controls.....	20.3	100	80	70	50	90	60
Cancer immune mice .....	47.7	86.6	100	73.3	66.6	86.6	60

*Experiment 2.*—Nineteen mice were injected with an immunizing dose of 0.3 cc. of defibrinated mouse blood. 10 days later these animals, with ten controls, were inoculated with a transplantable carcinoma. After 3 weeks, four of the nineteen immunized animals and nine of the controls had developed tumors. At this time all the immunized animals, including the four which had developed tumors, with ten normal mice as controls, were each inoculated intraperitoneally with 3 mg. of a 27 day old culture of bovine tubercle bacilli<sup>7</sup> taken up in 0.5 cc. of normal salt solution. The mice were kept throughout the experiment in individual jars. The distribution of the organisms at autopsy is shown in Table II. The average duration of life after inoculation for the control animals was 14.7 days, and for the cancer immune animals 24.3 days. The four animals which had been immunized but developed tumors lived on an average of 17.7 days. The rate at which the mice died is shown in Text-fig. 3.

<sup>7</sup> The same culture was used in this experiment as in the first, but in the interval it had been passed through an x-rayed guinea pig.



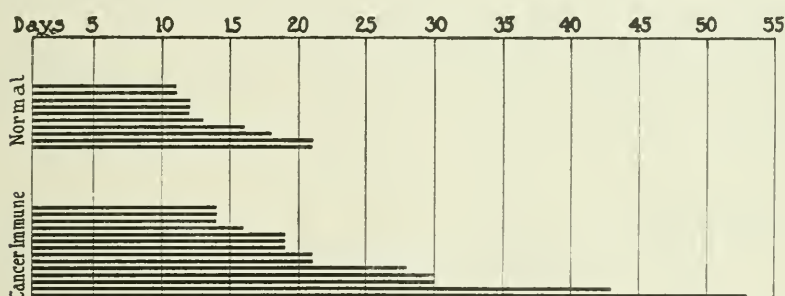


TEXT-FIG. 2. The horizontal lines show duration of life after inoculation of normal and cancer immune mice with tubercle bacilli. The average time of survival of the normal animals was 20.3 days, and of the cancer immune animals 47.7 days.

TABLE II.

*Distribution of Tubercle Bacilli in the Cancer Immune Mice and the Control Mice from Experiment 2.*

Group.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidneys.
	days	per cent	per cent	per cent	per cent	per cent	per cent
Controls.....	14.7	100	100	81.9	0.9	100	100
Cancer immune mice.....	24.3	100	100	73.3	13.3	80	73.3



TEXT-FIG. 3. The lines show the survival of individual mice after inoculation with tubercle bacilli. The normal animals averaged 14.7 days, while the cancer immune animals averaged 24.3 days.

The two experiments agree in showing that mice first immunized to, and then inoculated with a transplantable mouse cancer exhibit a greater resistance to bovine tuberculosis than do normal animals. On the other hand, we have noticed that while animals ineffectively immunized against cancer show a definite lymphoid reaction in the blood, it is less marked than when the immunity is perfect. In this connection, it is of interest to observe that the four mice of this kind in Experiment 2 outlived the controls but succumbed earlier than the completely immune ones. However, a larger series of animals would be needed to establish this point.

*Destruction of Lymphocytes in Cancer Immune Mice and Resistance to Tuberculous Infection.*

If the lymphocytic reaction in the cancer immune animals is the factor which determines the enhanced resistance to tuberculous

infection, the destruction of these cells by means of the x-ray should reduce resistance to a point even below that of normal animals. The following experiment was performed with this in view.

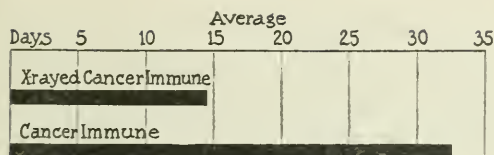
*Experiment 3.*—Thirty-eight mice were immunized with 0.3 cc. of defibrinated mouse blood, and 10 days later, with ten control animals, were inoculated with a transplantable mouse carcinoma. Five of the thirty-eight immunized animals developed cancers, as did all the controls. 2 weeks after the cancer inoculation, eighteen of the immune animals were given a daily dose of x-ray until they had received seven exposures. At this time, or 3 weeks after the cancer inoculation, both the x-rayed and non-x-rayed cancer immune mice were each inoculated intraperitoneally with 2 mg. of a culture of bovine tubercle bacilli. The same observations were made as in the two previous experiments. The distribution of the microorganisms is shown in Table III. The average duration of life after the tubercle bacilli inoculation in the x-rayed cancer immune animals was 14.5 days and in the non-x-rayed cancer immune animals 32.5 days (Text-fig. 4).

TABLE III.

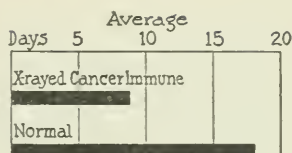
*Distribution of Tubercle Bacilli in the Organs of Animals from Experiment 3.*

Group.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidneys.
	days	per cent	per cent	per cent	per cent	per cent	per cent
X-rayed cancer immune mice .....	14.5	100	94.2	77.7	5.5	33.3	55.5
Intact cancer immune mice.....	32.5	95.2	80.9	66.6	4.7	28.5	33.3

*Experiment 4.*—Ten mice, which had been proved immune to cancer as a result of an injection of defibrinated blood, were given a series of doses of x-ray sufficient to reduce their lymphoid tissue. 3 weeks after the cancer inoculation, and 24 hours after the last x-ray exposure, the ten mice, with ten normals, were injected intraperitoneally with 3 mg. of a 3 weeks old culture of bovine tubercle bacilli taken up in 0.5 cc. of normal salt solution. All the animals at this time were in excellent physical condition. The same general routine was observed as in the former experiments. The x-rayed cancer immune animals died off very rapidly, the average duration of life being 8.9 days. The control mice died at a much slower rate and averaged 18 days of life after the inoculation; that is, they lived twice as long as the x-rayed series (Text-fig. 5).



TEXT-FIG. 4.



TEXT-FIG. 5.

TEXT-FIG. 4. The average duration of life of cancer immune mice subjected to repeated small doses of x-ray before inoculation with tubercle bacilli compared with the duration of life of intact cancer immune animals inoculated with the same infecting agent.

TEXT-FIG. 5. The average duration of life of cancer immune animals subjected to repeated small doses of x-ray before inoculation with tubercle bacilli compared with the duration of life of normal mice inoculated with the same infecting agent.

These experiments demonstrate conclusively that the enhanced resistance of cancer immune mice to tuberculous infection can be set aside or even reduced to a state of increased susceptibility by destroying the lymphocytes by means of exposure to x-rays.

#### DISCUSSION.

The experiments reported were not undertaken with the idea of establishing a relationship between cancer and tuberculosis, as we know of no sufficient reason to assume the existence of such a relationship. However, it has long been believed that some such specific antagonism between the two conditions exists. But considering that the ages at which cancer and tuberculosis reach their highest incidence are widely divergent, and that cancer rarely, if ever, attacks a debilitated individual, this idea of a specific antagonism would seem to have little basis in fact. That the two diseases may occur simultaneously in the same individual is borne out by many reports made in recent years.

It is probable that the lymphoid tissue of the body may be a determining factor in the phenomena of resistance in both tuberculosis and cancer, and yet the *modus operandi* involved may be absolutely different. Indications that the lymphoid cell plays a part in resistance to tuberculosis are numerous. The constant association of

these cells with the lesions of the disease is so well recognized that it need not be gone into here. In most of the clinical blood studies of tuberculous individuals, the white cells have been analyzed from the point of view of the polynuclear cell, but as these do not increase markedly, little attention has been paid to the other white cells. However, the blood counts show an interesting variation in the lymphoid cells which has not attracted the attention it merits. The total number of lymphocytes per c.mm. of blood in a normal adult varies between 1,500 and 2,500 cells. In one case of advanced pulmonary tuberculosis reported by Emerson,<sup>8</sup> there were only 530 lymphocytes, and in a case of miliary tuberculosis studied by the same author, the total number was only 227. Warthin<sup>9</sup> reports a case of miliary tuberculosis in which the lymphocytes made up only 5.5 per cent of a subnormal white cell count. On the other hand, in cases which give a good prognosis, these cells may form 40 to 50 per cent of a white cell count somewhat higher than normal, which means a substantial increase in the lymphoid cells. Wack<sup>10</sup> has observed that cases of tuberculosis advancing rapidly show a decrease, and those healing or healed a corresponding increase in the mononuclear cells. While the reports in the literature are not as convincing as might be desired, yet they present indications to the effect that the lymphocytes vary directly as the degree of resistance to the tuberculous infection. To bring together the evidence which bears on the participation of the lymphocyte in resistance to tuberculous infections, we have (a) its constant association with the pathologic lesions of the disease, (b) its fluctuation in the blood with the progress of the infective process, (c) the reduced resistance to the infection in animals depleted of their lymphoid tissue by means of x-ray, and (d) a marked enhancement of resistance to the infection displayed by animals with a high lymphocyte count resulting from the establishment of cancer immunity.

<sup>8</sup> Emerson, C. P., *Clinical Diagnosis*, Philadelphia and London, 4th edition, 1913, 624.

<sup>9</sup> Warthin, A. S., *Med. News*, 1896, lxxviii, 89.

<sup>10</sup> Wack, P., *Deutsch. Arch. klin. Med.*, 1914, cxv, 596.

## SUMMARY.

Mice so x-rayed as greatly to reduce the lymphoid tissue are rendered highly susceptible to tuberculous infection. On the other hand, when a marked lymphocytosis is induced by first immunizing mice against, and then inoculating them with cancer, the resistance to tuberculous infection is greatly enhanced. This heightened resistance may be set aside and even changed to a state of increased susceptibility to the infection by again depleting the lymphocytes by means of the x-ray.





# THE TRANSPLANTATION OF SPLENIC TISSUE INTO THE SUBCUTANEOUS FASCIA OF THE ABDOMEN IN RABBITS.

By O. T. MANLEY, M.D., AND DAVID MARINE, M.D.

(From the H. K. Cushing Laboratory of Experimental Medicine of Western  
Reserve University, Cleveland.)

PLATE 54.

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The reactions of certain structures of the spleen, for example the pulp cells and the endothelium of the sinuses, to many varieties of toxins have been extensively studied. On the other hand, little is known of the nature and function of the Malpighian bodies. While morphologically they resemble lymphoid tissue, the fact that this tissue rarely shows the reactions seen in other lymphoid tissues to bacterial toxins like typhoid, streptococcus, etc., may be of significance. In certain states, however, as in lymphatism, exophthalmic goiter, etc., this tissue, like the thymus, shares in the general lymphoid hyperplasia. Then too, its arrangement as an envelope about the arteries is not duplicated elsewhere in the body. The question of the relative importance of the structures in regeneration also has received little attention. Are the Malpighian bodies, the pulp cells, and the sinuses separate tissues with separate functions, or are they more interrelated functionally and morphologically than their anatomical appearances indicate? It occurred to us that transplantation, if this was possible, would throw some light on the subject of regeneration and possibly on the relative value of the tissues in this reaction. We have not been able to find any record of the transplantation of splenic tissue where the grafts were studied from these viewpoints or from the standpoints of the growth and permanence of the grafts.

TABLE I.  
*Transplantation of Spleen.*

Rabbit No.	Sex.	Date of splenectomy.	Date of thymectomy.	Date of transplantation.		First examination.		Final examination.		Histological examination.
				Autotransplantation.	Homoio-transplantation.	Day.	Condition.	Day.	Condition.	
1	Male.	1915 Aug. 26	1915	1915	1915 Dec. 16	56	—	56	—	Negative (infected); infiltrated with pus cells and edematous.
2	"	" 26	Nov. 29		Nov. 29	15	—	15	—	No trace of spleen transplant found.
3	Female.	" 26	" 29		Dec. 16	65	—	65	—	Negative at examination; area not removed.
4	Male.	Nov. 5	" 29		Nov. 29	17	—	17	—	Negative; nearly absorbed; marked connective tissue reaction and invasion; persistence of the spleen trabeculae; no lymph or pulp tissue present.
5	"	" 5	Dec. 2		Dec. 16	67	—	67	—	Complete absorption.
					" 18	63	—	63	—	Absorbed area with much yellow pigment in endothelial-like cells; some lymph tissue. This is clearly an atrophic spleen transplant with persistence of the endothelial elements.
6	Female.	" 5	" 2		" 2	14	+	14	+	Positive; marked connective tissue reaction and involution. Lymph and pulp tissues still present; highly vascular; in the process of absorption.
					" 16	65	—	65	—	Complete absorption. Several areas contain large numbers of phagocytic cells engorged with blood pigment.
7	Male.	" 5	" 2		" 16	65	—	65	—	Complete absorption.
8	"	" 5	" 3		" 16	67	—	67	—	Negative at examination; area not removed.
9	Female.	" 5	" 3		" 16	119	—	119	—	" " " " " "

10	Female.	Nov. 29	Nov. 29	Nov. 29	8	+	8	+	Positive; very well developed blood supply; marked connective tissue reaction.
11	Male.	Dec. 2	Dec. 2	Dec. 2	11	+	11	+	Positive; well organized blood supply; one large trabecula; both lymph and pulp cells present; well marked connective tissue reaction.
12	"	"	"	"	13	+	80	-	Negative at examination; area not removed.
13	"	"	"	"	13	+	13	+	Positive; in the process of absorption; marked connective tissue reaction, with ingrowth into transplant area; good blood supply.
14	Female.	"	"	"	Dec. 16	-	67	-	Negative at examination; area not removed.
		"	"	"	67	++++	325	++++	Transplant 4 by 2.5 by 2 mm.; whole transplant shows usual splenic vascularity; encapsulated; typical Malpighian body formation, trabeculae, pulp, and sinus formation.
15	"	"	"	"	65	-	65	-	Complete absorption.

Many experiments of dislocation, often misnamed transplantation have been reported, as the experiments of Hédon<sup>1</sup> of pulling the organ through an abdominal wound with the blood supply intact, and suturing it into the subcutaneous tissues. Those of Lüdke,<sup>2</sup> of introducing bits of spleen into the spleens of alien species of animals, are not related to our problem, since heterotransplantation in mammals has never succeeded. The blood vessel suture experiments of Carrel<sup>3</sup> also have no bearing on the questions suggested above.

Brief mention of our first observations with spleen transplantation was made in a previous paper.<sup>4</sup> At that time only negative results with both auto- and homoiotransplantations had been obtained.

#### EXPERIMENTAL.

We now wish to report the end-results of a series of transplantations made more than a year ago. Twelve attempts at homoio-transplantation and six attempts at autotransplantation were made on fifteen rabbits. The more important data relating to each experiment are given in Table I.

#### *Method.*

The method employed is the same as that used by us in the transplantation of ductless gland tissues, and consists of transferring small sections of the spleen of about 2 mm. in their greatest dimension under strict aseptic precautions. Alcohol and bichloride of mercury were used instead of iodine for skin sterilization, because it is necessary to control the intake of iodine whenever the thyroid may be involved directly or indirectly in the experiment. After making a transverse abdominal skin incision about 2 cm. in length, the subcutaneous fascia is lifted with fine forceps, punctured with a cataract knife, the tissue introduced, and the fascial opening closed with a black silk ligature, which also serves to mark the site for subsequent examinations.

<sup>1</sup> Hédon, E., Transplantation sous-cutanée de la rate, *Compt. rend. Soc. biol.*, 1899, vi, 560.

<sup>2</sup> Lüdke, H., Ueber Milztransplantationen, *Münch. med. Woch.*, 1909, lvi, 1469.

<sup>3</sup> Carrel, A., Remote Results of the Reimplantation of Kidney and Spleen, *J. Exp. Med.*, 1910, xii, 146.

<sup>4</sup> Manley, O. T., and Marine, D., Transplantation of Ductless Glands with Reference to Permanence and Function, *J. Am. Med. Assn.*, 1916, lxxvii, 260.

The spleen was completely removed at the time of transplantation in the group of autotransplants, while in all the homoiotransplants it was removed some time (from 112 to 24 days, in the majority about 40 days) before transplantation. This is probably too long a time interval to include a possible advantage to the transplant that might accrue from a splenic insufficiency, since Musser and Krumbhaar<sup>5</sup> and others<sup>6</sup> have shown that certain animals (dogs) usually begin to recover from the systemic effect of splenectomy, as indicated by the erythrocyte counts, in 3 to 4 weeks.

It has been definitely established that a physiological insufficiency markedly influences the growth of autotransplants of the ductless glands, but it does not influence to any extent the taking of the transplants. Likewise with homoiotransplants there is no evidence that an induced physiological insufficiency modifies the taking. Sex also does not influence the taking, growth, or rate of destruction of the transplanted tissue. The thyroid was removed in every instance but one at the time of, or shortly before transplantation without any influence on the taking or growth of the spleen grafts. Removal of the thyroid, ovaries, and spleen at one time, with immediate autotransplantation in one animal, was without effect.

### *Homoiotransplantation.*

Direct and microscopic examinations were made in three cases on the 14th, 15th, and 17th days, while in the remaining ones it was delayed for 2 or more months. Only in one instance, the 14 day transplant, was there definite splenic tissue remaining, and in this only the lymphoid tissue and trabeculae could be recognized. The one outstanding difference between the spleen and other tissue transplants is the marked early connective tissue reaction resembling

<sup>5</sup> Musser, J. H., Jr., and Krumbhaar, E. B., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. VI. The Blood Picture at Various Periods after Splenectomy, *J. Exp. Med.*, 1913, xviii, 487.

<sup>6</sup> Sollberger, H., Beiträge zur Physiologie der Drüsen. XIX. Fortgesetzte Beiträge zur Lehre von der Funktion der Milz als Organ des Eiweissstoffwechsels. Über die Kompensationsvorgänge nach Milzexstirpation, *Biochem. Z.*, 1913, lv, 13.



the granulation tissue formation seen in chronic inflammation. Infection from organisms in the spleen, however, is not probable, as only normal spleens from rabbits with no evidence of acute infection were used. It is more probable that the splenic tissue when transferred to the subcutaneous fascia is an active irritant. The studies of Carrel<sup>7</sup> on the effect of tissue extracts on the growth of connective tissue *in vitro* are of interest in this connection. He found that adult spleen extracts, thyroid extracts, and the Rous chicken sarcoma extract markedly accelerated the proliferation of connective tissue. Our observations on the irritant effect of spleen grafts confirm his observations. The effect is present in auto- as well as homoiografts, though perhaps the homoiografts excite a slightly greater connective tissue reaction. Carrel also found that thyroid extracts excite in the living animal an even more marked connective tissue proliferation both in healing skin and periosteum wounds. We have been unable to detect an excessive connective tissue proliferation around thyroid grafts. It would seem, as suggested by Carrel, that spleen extracts could be used to promote the granulation of wounds.

#### *Autotransplantation.*

Four transplants were examined on the 8th, 11th, and 13th days, respectively. All were positive and seemingly active. The 8th, 11th, and one 13th day transplants were removed for histological examination. In contrast with the homoiotransplants, examined after approximately the same period, all were positive with well established blood supplies and central necrosis, and the peripheral zone of pulp, lymphoid, and trabecular tissues was distinct. There was also the same connective tissue reaction seen in the homoio-transplants.

Of the three transplants examined at the 65th, 67th, and 80th days, complete absorption leaving large white scars at the sites had occurred in two, while in the third (Rabbit 14) a dark red, sharply circumscribed mass appeared, the size of a small wheat kernel. There were several large vessels entering it, and except for its darker color it could

<sup>7</sup> Carrel, Artificial Activation of the Growth *in Vitro* of Connective Tissue, *J. Exp. Med.*, 1913, xvii, 14.

easily have been mistaken for an autothyroid transplant. We did not remove it, and it was recovered at autopsy 325 days after transplantation, the rabbit having died of acute pneumonia and pleurisy. Grossly, the transplant measured 4 by 2.5 by 2 mm., and was dark bluish red in color. It had evidently reached its maximum growth before the first examination on the 67th day, since it was not noticeably larger on the 325th day than at the first examination. This could be interpreted as evidence that its growth had occurred in response to a physiological insufficiency during the time when other tissues were assuming the function lost through removal of the spleen.

Microscopic examination reveals typical congested splenic tissue embedded in striped muscle and mammary gland. The capsule is very thick as compared with transplants of ovary, parathyroid, thyroid, or adrenal, and several connective tissue bands extend from the capsule to the surrounding muscle fascia. Many large vessels run in the outer layer of the capsule. Small trabeculae also extend throughout the gland parenchyma. Their numbers are the same that one finds in the normal spleen, while their size is proportional to the size of the organ. No attempt was made to demonstrate the presence or absence of smooth muscle fibers in the capsule and trabeculae and no studies on the reticulum have been made. In some sections one can see as many as sixteen well defined Malpighian bodies with the characteristic central artery and radial capillary system. These Malpighian bodies are surrounded by typical splenic pulp with large highly congested sinuses. In places there are deposits of the yellowish brown blood pigment usually seen in the normal spleen pulp. We have, therefore, to deal with a small newly formed spleen developed in an entirely foreign field as regards its location, nerves, and blood vessel relations. It has all the characteristics of the normal spleen in as far as these have been investigated, both as to the number of its component structures, capsule, trabeculae, Malpighian bodies, pulp, sinuses, and blood pigment, and their relation to each other. The trabeculae are proportional to the size of the organ, while all the other structures are of normal proportions, suggesting that the trabeculae play a purely mechanical part. The capsule is still relatively much thicker than that of the normal spleen,

and it seems probable that this is due to the enormous proliferation of the connective tissue which takes place around the graft very early (within 2 weeks), and also that this tends to return to relatively more normal proportions through absorption as the connective tissue becomes adjusted to its new neighbor. One of the striking facts, then, is that the regeneration involves all the structures in such a way that both their normal proportions and arrangements are wholly retained. This would suggest a fairly uniform vitality for all the component parts as well as a close functional interrelation. It may be recalled that in the adrenal the cortical cells readily survive, while the medullary cells invariably die, at least in our experience. When one recalls that in the thyroid only a narrow peripheral zone of three or four cells in thickness survives the transfer and that the whole interior portion undergoes necrosis, it is of much more significance that a fully regenerated organ should develop after transplantation of splenic tissue than in the case of such tissues as the thyroid or parathyroid which contain but a single specialized tissue.

The regeneration of Malpighian bodies is also of interest. Why does the lymphoid tissue not regenerate around the regenerated arteries running in the capsule and trabeculae as well as in those of the interior of the lobule? The best studies on the taking of engrafted tissues indicate that the host supplies the new blood vessels to the transferred tissue rather than that it utilizes the possible surviving fragments in the graft. It is difficult to harmonize this with the known fact that the intralobular arteries of even a regenerated spleen develop this envelope of lymphoid-like cells. Either the vessels must be specific, which does not seem probable, or the lymphoid-like cells are specific in that they control and determine the blood vessel arrangement. If this is the case, we have proof that the Malpighian bodies represent a different type of lymphoid tissue, functionally as different from ordinary lymph node tissue as is thymic tissue.

The same questions must also be raised in connection with the development of the spleen sinuses as have been discussed in connection with the formation of the Malpighian bodies. The careful study of an appropriate series of these grafts will doubtless give valuable data, both as to the normal development and function of this peculiar

organ. The survival and growth of the pulp cells are more easily understood and the process is probably similar to that of other single type tissues.

The general features of the transplant are shown in the accompanying photomicrographs (Figs. 1, 2, and 3).

#### SUMMARY.

We have not found in the literature a report of an instance of permanent homoio- or autotransplantation of the spleen, or of the probably closely related spleno- and hemolymph glands. Spleen autotransplants with considerable difficulty as compared with thyroid, parathyroid, ovary, or adrenal cortex. This may be due to its complex anatomical structure. An instance of a permanent autotransplant has been observed. None of our attempts to homoio-transplant it were successful beyond the usual taking and persistence for 2 or 3 weeks, common to all homoiografts. The successful permanent subcutaneous autotransplantation had all the morphological characteristics of a fully differentiated and functionally active spleen. This method of transplantation would seem to offer a means of learning more of the normal development, regeneration, and function of this complex tissue.

#### EXPLANATION OF PLATE 54.

FIG. 1. Photomicrograph of the 325 day spleen transplant (Rabbit 14), showing adjacent abdominal muscle, capsule, and general characteristics.  $\times 20$ .

FIG. 2. Photomicrograph of the same transplant, showing the structure.  $\times 100$ .

FIG. 3. Photomicrograph of the same transplant, showing trabeculæ, pulp, and a Malpighian corpuscle.  $\times 100$ .





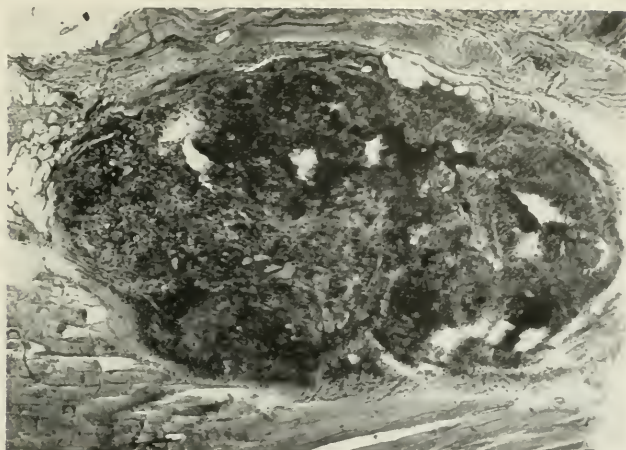


FIG. 1.

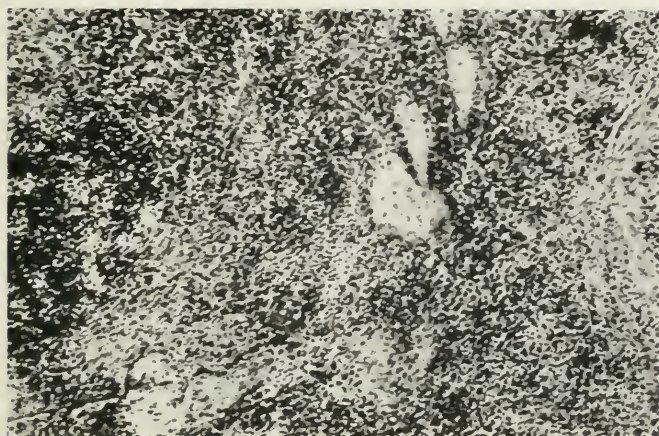


FIG. 2.

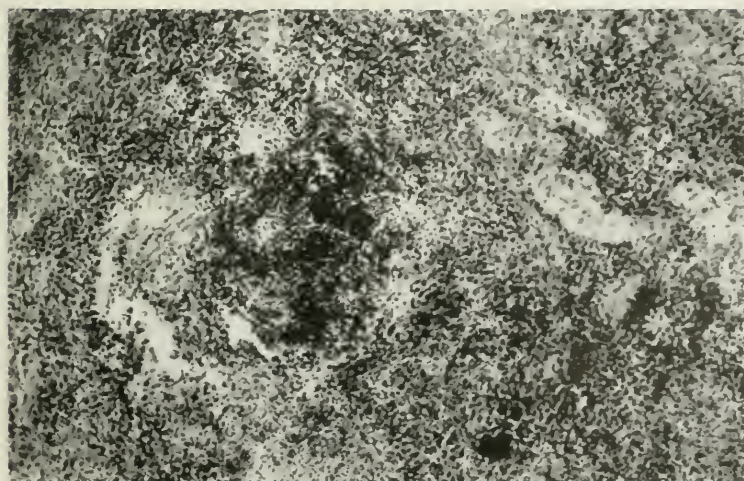


FIG. 3.

(Manley and Marine: Transplantation of Splenic Tissue.)





## A NOTE ON THE PRODUCTION OF ANTIPNEUMOCOCCUS SERA.

BY AUGUSTUS B. WADSWORTH, M.D., AND MARY BUTLER KIRKBRIDE.

(From the Division of Laboratories and Research of the New York State Department of Health, Albany.)

(Received for publication, January 5, 1917.)

The value of homologous antipneumococcus serum as a therapeutic agent in lobar pneumonia caused by Type I pneumococci, has been demonstrated at The Rockefeller Institute Hospital during the past 3 years. It is desirable that the serum should be brought within reach of a larger section of the community than has been possible hitherto, but difficulties in production and administration of the serum have made this impossible. The present report deals entirely with the production of antipneumococcus sera.

In August, 1915, immunization of horses to pneumococci of the Types I, II, and III was commenced at the farm of the New York State Department of Health near Albany, under unfavorable conditions owing to inadequate facilities at the laboratory and the farm, and to irregularities in the injections caused by bad and occasionally impassable roads and by exposure of cultures to intense cold during the winter months. None of the horses immunized was under 11 years old. It was therefore thought that if potent sera could be produced under these adverse conditions, the success would strengthen the belief in these methods of immunization, and suggest possibilities for their broader application.

For immunization the method of Cole<sup>1</sup> with washed culture sediment and the method of Wadsworth,<sup>2</sup> using whole fresh 18 hour cultures, were followed. Beginning with weekly injections, or injections on 3 successive days each week, of heated cells or cultures, these were later followed by increasing doses of living organisms.

<sup>1</sup> Cole, R., *J. Exp. Med.*, 1912, xvi, 644.

<sup>2</sup> Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 54, 78.

The potency of the sera was tested by protection tests on mice and by agglutination reactions. Intraperitoneal injection of control mice with 0.000001 cc. of the standard strains of Type I, II, or III, received from The Rockefeller Institute Hospital, and used for immunization and for these tests, was invariably fatal in less than 40 hours. Of three horses immunized against Type I organisms, 0.1 cc. of serum from No. 26 (culture sediment) or No. 28 (whole culture) protected, as a rule, against 0.4 cc., and not infrequently against 0.5 cc. of homologous culture. In three tests where 0.6 cc. of culture was used, the mice did not survive. 0.1 cc. of serum from No. 27 (culture sediment) protected, as a rule, against 0.2 cc., and sometimes against 0.3 cc. of homologous culture.<sup>3</sup> Wide variations similar to those reported by other workers were shown in the protection tests with Type II sera. 0.1 cc. of serum from No. 16 (culture sediment) or from No. 32 (whole culture) protected at times against 0.1 and 0.01 cc. of culture, and quite constantly against 0.001 cc.; a slightly lower protective titer was given by serum from Horse 29 (culture sediment).

Agglutination reactions indicated an inverse relation between the agglutinating power and the protective value of the sera, as in each type the horses with a lower protective index gave serum having a higher agglutination titer. Owing to the wide range of variations in agglutination reactions, due to differences in the density of the pneumococcus suspensions, satisfactory comparison with previous agglutination tests made by other workers is practically impossible.

While successful active immunization of rabbits, sheep, and goats with Type III organisms (*Pneumococcus mucosus*) has been reported, the production of sera of sufficient potency to agglutinate fresh untreated homologous organisms or to confer passive immunity on mice has apparently been unsuccessful hitherto. These failures have been considered to be due to the large mucoid capsule and tenacious slimy material produced by and surrounding the organ-

<sup>3</sup> The serum from Horse 27 has since protected 0.1 cc. against 0.4 cc. of culture. Experimental tests of different methods for standardizing the potency of antipneumococcus sera are in progress. Meanwhile in our experience more satisfactory results are obtained when 0.1 cc. of Type I serum instead of 0.2 cc. is used in protection tests on mice.

isms, which necessitated preliminary treatment, such as with a weak acid, according to the method of Porges,<sup>4</sup> before agglutinations could be obtained in the homologous sera.

For purposes of study and comparison, the immunization of a horse to Type III organisms (culture sediment) was carried on in conjunction with the horses of Types I and II.<sup>5</sup> The strain used for immunization while at first less virulent soon reached that of the other standard types, 0.000001 cc. killing a mouse within 40 hours. Protection tests showed that the serum possessed a definite though slight and variable protective action for mice. 0.2 cc. of serum in one instance protected against 0.001 cc. of culture, while in other tests mice receiving the same amounts of serum and culture died in 35, 63, or 70 hours. The same tests with several other Type III strains gave similar results.

In agglutination tests with fresh untreated Type III organisms, in concentrations of 1:1, agglutination was prompt and striking, usually showing before the tubes were placed in the water bath. Within 15 to 30 minutes agglutination was generally complete, and a loose cap had formed similar to those often seen in tests with Type I and Type II organisms and their homologous cultures. While agglutination in concentrations of 1:1 and 1:10 occurred as promptly as with Type I and Type II in their homologous sera, it was entirely absent in dilutions above 1:40. Undiluted serum obtained 5½ months after the last immunizing injection agglutinated Type III organisms with equal promptness; serum of 1 month later, however, caused no reaction. This is of interest because the abscess which had been discharging during the previous six months had healed meanwhile. All strains of the *mucosus* type tested, which were morphologically and culturally typical, agglutinated promptly with

<sup>4</sup> Porges, O., *Wien. klin. Woch.*, 1905, xviii, 691. von Eisler, M., and Porges, O., *Centr. Bakteriolog., 1te Abt., Orig.*, 1906, xlii, 660. Hanes, F. M., *J. Exp. Med.*, 1914, xix, 38.

<sup>5</sup> During the course of immunization a large abscess formed at the site of injection from which Type III organisms were isolated. Later a second abscess of a similar nature developed between the shoulder blades. It would be of interest to know the connection, if any, between these local foci of infection and the exceptional potency which the serum later developed.

one exception, but several strains which did not dissolve in bile failed to agglutinate. In a number of instances Type III organisms, which might readily have been classified as Type IV, owing to apparent absence in inoculated mice of the characteristic sticky peritoneal exudate and to the unusually small capsule of the organisms, were agglutinated promptly in Type III serum. Conversely, absence of agglutination in Type III serum made possible prompt exclusion from the Type III group of pneumococci obtained from the peritoneum of mice, in which a slightly viscous exudate was apparently present. The serum is, therefore, of distinct value in the diagnosis of pneumococcus types and should be used as a routine procedure together with the sera of Types I and II.

#### SUMMARY.

Horses immunized to Type I pneumococci developed serum, 0.1 cc. of which protected against 0.5 cc. of a virulent culture, 0.000001 cc. of which killed mice in less than 40 hours. Protective tests of serum from horses immunized to Type II organisms varied, 0.1 cc. protecting, however, in certain instances against 0.1 and 0.01 cc. of virulent homologous culture. Types I and II sera obtained in our experiments with culture sediment and whole culture did not vary markedly for a given type and corresponded closely in their protective titer with samples of sera received from The Rockefeller Institute Hospital. It is therefore evident that the following minimum standard of 0.1 cc. of serum to protect mice against at least 0.2 cc. of virulent cultures can and should be maintained when serum is to be used for the treatment of cases. By further study and comparison of these different methods of immunization it is hoped that sera of greater potency may be produced, but as yet this has only been accomplished in exceptional instances.

A horse immunized with Type III (*Pneumococcus mucosus*) developed serum having a slight degree of protection for mice against the corresponding organisms. This serum was sufficiently potent, however, to cause prompt and complete agglutination when combined with fresh untreated homologous organisms, thus avoiding the preliminary treatment to remove the capsule which has previously been held necessary. As a diagnostic aid in the differentiation of pneumococcus strains, the serum has proved of distinct value.

## EXPERIMENTAL STUDIES UPON LYMPHOCYTES.

### I. THE REACTIONS OF LYMPHOCYTES UNDER VARIOUS EXPERIMENTAL CONDITIONS.

By ALWIN M. PAPPENHEIMER, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.)

(Received for publication, January 6, 1917.)

Hunger, chronic inanition, infections, or such specialized forms of injury as those caused by the x-ray, all bring about a sudden or gradual destruction of the small thymus cells. We have no knowledge as to the significance of the fragility of thymus lymphocytes, nor is anything known of the more intimate factors concerned. Compared with the detailed studies bearing upon the biological behavior of the red blood cells, our knowledge of the thymus cells and of the reactions of lymphocytes in general to environmental conditions is meager.

The fact that we have had no sharp criterion of injury, comparable to the hemolytic reaction, and available for comparative quantitative studies, is probably one of the reasons for this. Morphological changes in film preparations or tissues are often difficult to interpret. The cessation of ameboid activity, which has been used by Christian and Leen<sup>1</sup> and others as an index of cell injury, in the study of leukotoxins, is hardly applicable in the case of the sluggish and often entirely immobile lymphoid cell.

Since the introduction into common use of trypan blue and other vital stains, it has been repeatedly observed that the injured or dead cell reacts to these dyes by a diffuse staining of the entire cell, including the nucleus. Advantage has been taken of this fact by Gross<sup>2</sup> in his work on experimental nephritis, by

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<sup>1</sup> Christian, H. A., and Leen, T. F., Some Further Observations on Leucocytotoxins, *Boston Med. and Surg. J.*, 1905, clii, 397.

<sup>2</sup> Gross, W., Experimentelle Untersuchungen über den Zusammenhang zwischen histologischen Veränderungen und Funktionsstörungen der Nieren, *Beitr. path. Anat. u. allg. Path.*, 1911, li, 528.



MacCurdy and Evans<sup>3</sup> in their studies of the cytopathology of experimental poliomyelitis, and by Rous and Jones<sup>4</sup> in their work on the protection of pathogenic organisms by living tissue cells. Evans and Schulemann<sup>5</sup> state that mechanical injury to leukocytes suspended in trypan blue causes instant diffusion of the dye into the traumatized cells.

It occurred to us that use could be made of this diffuse staining of injured cells to test the effect of various agencies upon the lymphocytes *in vitro*. A simple and satisfactory technique has been developed to this end. Our studies have been concerned chiefly with the small thymus cells of the rat, which provide readily available material, but we have applied the method also to lymphocytes from freshly removed human tonsils.

The method of procedure is as follows: The freshly excised thymus is teased with small forceps, the rat having been previously exsanguinated by aspirating blood from the heart, under ether anesthesia. There is obtained in this way a milky suspension composed entirely of small thymus lymphocytes, and practically free of red blood cells and fixed reticular elements. Suspensions of tonsil lymphocytes are prepared in a similar way, although it is more difficult to obtain them wholly blood-free.

The cell suspensions are then subjected to various experimental influences, and after a fixed period, a solution of trypan blue in Locke's solution is added in known dilution, which, however, may be purposely varied to meet different conditions. When the cells were suspended in Locke's fluid, a dilution of 1:10,000 gave the best results; in the presence of serum or other colloids, a dilution of 1:5,000 is preferable. A drop of the stained suspension is then placed in a counting chamber, and the percentage of stained cells immediately determined by counting from 300 to 600 cells with the low power.

There is generally no difficulty in detecting with No. 3 objective the sharply stained, non-refractile blue cells from the highly refrac-

<sup>3</sup> MacCurdy, J. T., and Evans, H. M., Experimentelle Läsionen des Centralnervensystems, untersucht mit Hilfe der vitalen Färbung, *Berl. klin. Woch.*, 1912, xlix, 1695.

<sup>4</sup> Rous, P., and Jones, F. S., The Protection of Pathogenic Microorganisms by Living Tissue Cells, *J. Exp. Med.*, 1916, xxiii, 601.

<sup>5</sup> Evans, H. M., and Schulemann, W., The Action of Vital Stains Belonging to the Benzidine Group, *Science*, 1914, xxxix, 443.

tile unstained lymphocytes. Under certain conditions, however, various degrees of transitional staining are encountered which make the determinations uncertain. This is particularly the case when the proportion of stained cells is high. There is then a tendency for all the cells to become gradually stained, so that a repetition of the count may give a much higher proportion of stained cells. On the other hand, when the proportion of stained cells is low, the time element becomes a less important factor.

Although these variations are at times disturbing and make it difficult to obtain coherent results, the method has proven of service, and by following a routine mode of procedure, the accidental fluctuations have been greatly reduced. We have repeatedly obtained duplicate counts varying less than 1 per cent, and in general the variations inherent in the method probably do not exceed 5 per cent, except when the proportion of stained cells is high.

Using this technique, we have systematically studied the reactions of the thymus cells under a fairly wide range of experimental conditions. The primary purpose was to test the availability of the method, so that our observations in many cases are merely the starting-point for a more thorough study of the questions involved.

#### *The Effect of Hypertonic and Hypotonic Solutions.*

Suspensions of thymus and tonsil lymphocytes were exposed to salt solutions of various concentrations, and the percentage of stained cells after the addition of trypan blue was determined. Locke's fluid was rendered hypotonic by the addition of distilled water, and the same test was applied.

The optimum concentration was found to be between 0.8 and 0.9, an increase in the percentage of stained cells being noted both with hyper- and hypotonic solutions. Hypotonic solutions of Locke's fluid were better borne than corresponding dilutions of pure sodium chloride.

#### *The Effect of Varying Hydrogen Ion Concentration.*

Fragments of thymus from the anesthetized rat were teased in balanced solutions of 0.093 M  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ , the hydrogen

ion concentration being varied from  $P_H$  8.0 to  $P_H$  6.0. The isotonicity of the solutions was tested against rat blood corpuscles. The percentage of stained cells upon adding trypan blue was determined after 1 to 2 hours. The time factor was checked, as far as possible, by counting the extremes of the series first, so that variations due to the longer exposure would affect especially the cells in solutions of approximately the hydrogen ion concentration of the blood.

Three repetitions of the experiment gave substantially the same result. The curves obtained, while not mathematically exact, probably represent fairly well the actual conditions. The optimum hydrogen ion concentration, or the concentration at which the minimum number of cells show diffuse staining, lies between  $P_H$  6.8 and  $P_H$  7.2. The variations between  $P_H$  7.0 and  $P_H$  8.0 are very slight, so we may infer that the fluctuations occurring during life in the circulating blood do not greatly affect the thymus cells. It is interesting to note that the optimum concentration,  $P_H$  6.8 to  $P_H$  7.2, is decidedly more acid than that of the circulating blood, but corresponds with the hydrogen ion concentration of the tissues as estimated by Michaelis.<sup>6</sup>

Beyond  $P_H$  6.8, there occurred in all the experiments a rapid increase in the percentage of stained cells. At  $P_H$  6.0 or  $P_H$  6.2, practically all the cells stain diffusely. At the alkaline end of the series plasmolysis of the cells occurred and the counting of the cells became difficult. The rise in the percentage of stained cells is not so marked as with the acid solutions and was not obtained at all in one of the experiments.

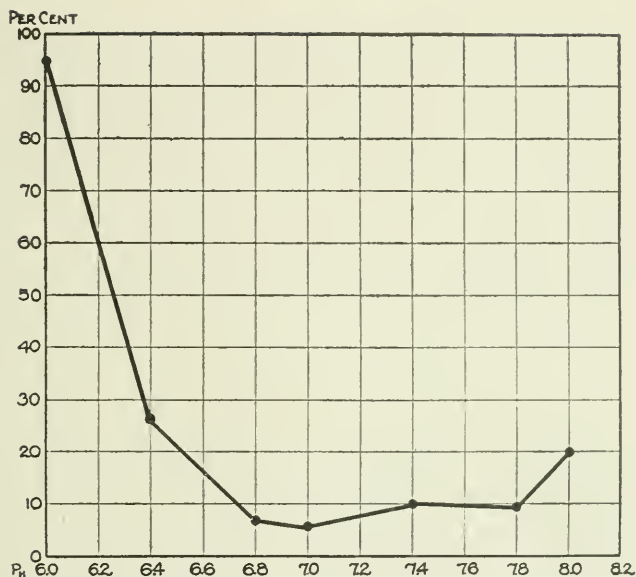
Text-figs. 1 and 2 are typical of the results obtained.

#### *The Effect of Temperature.*

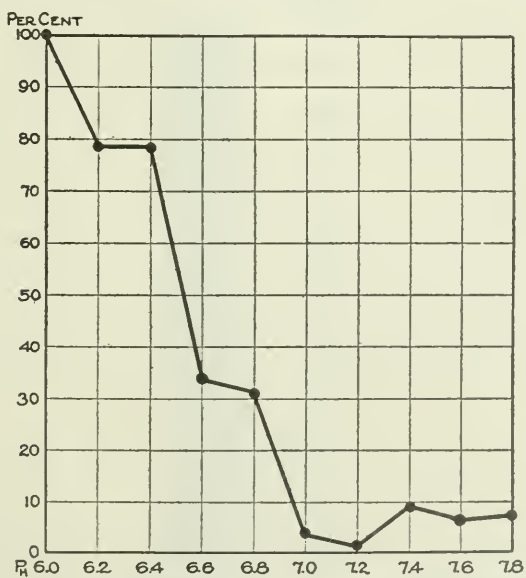
The effect of temperature was tested by immersing suspensions of thymus cells successively in small test-tubes in a water bath. Each tube was kept for 10 minutes at the desired temperature, and the proportion of stained cells immediately counted (Text-fig. 3).

It is seen that between 36° and 48°C. there is little variation in the percentage of stained cells. At 48°C. there is an abrupt rise in the curve, and at 51°C. practically all the cells are stained. The

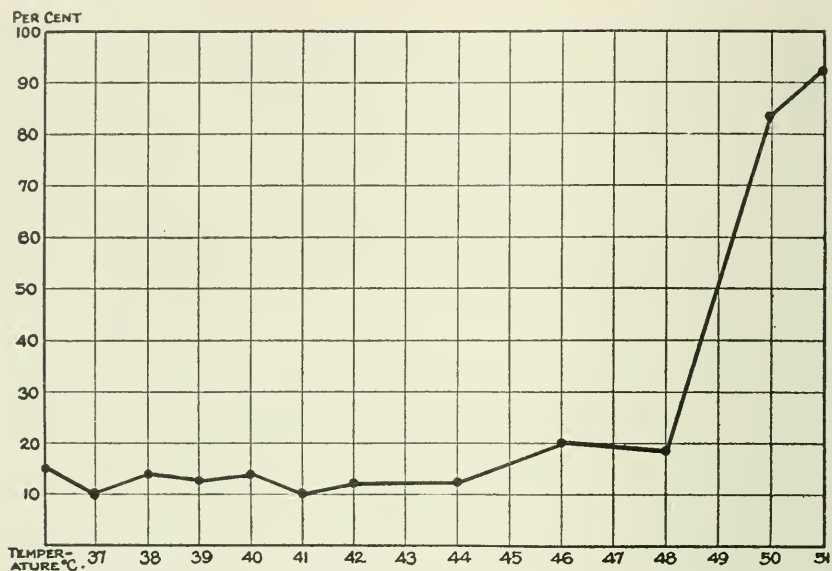
<sup>6</sup> Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914.



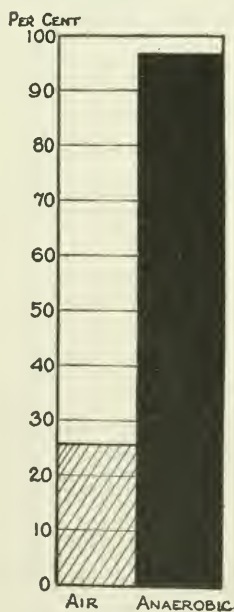
TEXT-FIG. 1. Experiment 1. The effect of phosphate solutions of varying hydrogen ion concentration upon the stainability of rat thymus cells.



TEXT-FIG. 2. Experiment 2. The effect of phosphate solutions of varying hydrogen ion concentration upon the stainability of rat thymus cells.



TEXT-FIG. 3. Experiment 3. The effect of temperature upon the stainability of rat thymus cells.



TEXT-FIG. 4. Experiment 4. The effect of asphyxia upon the stainability of rat thymus cells.

cells become irregular in shape and stain intensely. There is no diminution in their number. The curve suggests that the thermal death point lies between 48° and 51°C.

We have found no reference to the effect of heat upon lymphocytes, but Schultze<sup>7</sup> gives 50°C. as the temperature at which leukocytes (polynuclears?) undergo heat rigor.

Cells left over night in the ice box (temperature approximately 5–6°C.) show little if any increase in the proportion of stained cells. A detailed study of the effect of cold has not been made.

### *The Effect of Asphyxia.*

Suspensions of rat cells were made in previously boiled Locke's solution, and covered with a layer of albolene. These were then placed in the thermostat, and compared with a similar suspension which had been exposed to the air. The asphyxia thus produced gives rise to a notable increase in the percentage of stained cells (Text-fig. 4). A similar effect was brought about by exhausting the oxygen with pyrogalllic acid and sodium hydroxide.

If carbon dioxide gas is passed through a suspension of cells, flocculation occurs, the cells sinking in clumps to the bottom and leaving the supernatant fluid clear. The clumps, however, are easily broken up, and if the stream of carbon dioxide is suspended, the emulsion after being shaken resumes its normal appearance. The proportion of stained cells rises slowly, and is higher than that of a control suspension through which air is passed at about the same rate.

Pure oxygen passed through a suspension of cells brings about their rapid destruction. It was first thought that this might be due to trauma caused by the mechanical agitation; but the control in which air is allowed to bubble through vigorously shows that this is not the case.

### *The Protective Action of Serum and Other Colloids.*

The addition of serum, even in relatively dilute amount, greatly lowers the percentage of stained cells, and in sufficient concentra-

<sup>7</sup> Schultze, M., quoted by Marchand, F., in Krehl, L., and Marchand, F., *Handbuch der allgemeinen Pathologie*, Leipsic, 1908, i, 50.



tion may completely inhibit the staining with the ordinary strength of trypan used. For this reason, in order to bring out differences where sera are being used, it is necessary to add trypan in a concentration of 1:5,000 or more.

The following experiment is an example of the inhibitory action of serum.

Tube.	Mixture.	Percentage of stained cells.
1	Locke's solution, 0.5 cc. Thymus cells, 3 gtt.	20.6
2	Locke's solution, 0.5 cc. Thymus cells, 3 gtt. Rat serum, 3 "	None.

Various organic and inorganic colloids were tested as to their ability to prevent staining. It was found that gelatin (3 gtt. of a 10 per cent solution) completely inhibited the penetration of the dye. Egg albumin conferred slight protection; starch and gum arabic were without effect in the concentrations tried. Hemoglobin obtained by laking washed cells in distilled water and rendering the solution isotonic with sodium chloride was also found to be inert. A cholesterol emulsion was made by dissolving pure cholesterol in acetone, pouring the solution into distilled water, and removing the acetone by boiling. The opalescent fluid showed no visible particles. After being made isotonic with sodium chloride, a thick suspension of thymus cells was added and the stainability of the cells compared with a suspension in pure salt solution. No protective action on the part of the cholesterol was observed.

The inorganic colloids tested were arsenious sulfide and colloidal iron. The former in stronger concentrations caused agglutination of the cells and was precipitated about them. In weaker dilutions it appeared to act like serum and gelatin in affording protection against staining.

Colloidal iron was precipitated and the cells were agglutinated in all the concentrations tried.

Extraction of serum with chloroform does not affect its protective powers.

*The Effect of Certain Photodynamic Substances.*

*Hematoporphyrin.*—The solution contained 1 mg. per 1 cc. dissolved in 95 per cent alcohol.

The toxic effect of this substance, when activated by direct sunlight, is shown in the following experiment.

Tube.	Mixture.	Treatment.	Percentage of stained cells.
1	Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	Sunlight, 1 hr.	19.0
2	0.10 per cent ethyl alcohol in Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	" 1 "	15.6
3	Hematoporphyrin, 1 : 100,000 in Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	Darkness, 1 "	15.0
4	Hematoporphyrin, 1 : 100,000 in Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	Sunlight, 1 "	99+

The cells which had been exposed to the action of hematoporphyrin in direct sunlight, in a dilution of 1 : 100,000<sup>8</sup> showed immediate staining upon the addition of trypan blue. The effect of sunlight alone or of the alcohol is negative, as shown in Tubes 1 and 2.

*Chlorophyll.*—An impure solution of chlorophyll, obtained by extracting cabbage leaves with 95 per cent ethyl alcohol, and diluted 1 : 100 with Locke's solution, was tested against rat thymus cells as follows:

Tube.	Mixture.	Treatment.	Percentage of stained cells.
1	1 per cent chlorophyll in Locke's solution, 1.0 cc. Thymus cells, 6 gtt.	Darkness, 30 min.	26
2	1 per cent chlorophyll in Locke's solution, 1.0 cc. Thymus cells, 6 gtt.	Arc-light, 30 "	68

Exposure of the serum alone to the action of these photodynamic substances did not impair its subsequent protective power for the cells.

<sup>8</sup> The same solution of hematoporphyrin causes complete hemolysis of washed rat erythrocytes in 1 : 100,000 dilution, the controls kept in the dark remaining unlysed.

*The Effect of Exposure to X-Rays upon the Stainability of the Lymphocytes.*

Because of the known susceptibility of the thymus cells to x-rays, we tested the value of trypan as an indicator of cell injury by exposing small tubes containing suspensions of cells to radiation.<sup>9</sup> The control tubes were unexposed, and we were not informed until the end of the experiment which of the tubes had been subjected to the x-rays.

No significant differences could be detected as regards the number of stained cells in the tubes which had been irradiated and the controls. In one of the experiments hourly counts were continued for 7 hours, at which time the percentage of stained cells had risen in both tubes so as to make the counts unreliable.

That this seemingly negative result was probably due to the existence of a latent period is indicated by the following experiment.

*Experiment 5.*—Two healthy young rats of approximately equal weight and age were etherized, and a small portion of thymus was resected. Cell suspensions in Locke's solution were immediately counted with the addition of trypan blue.

Rat 1 gave 5.9 per cent of stained cells.

" 2 " 6.3 " " " " "

This showed that the thymus cells of the two rats before exposure were almost identical in their reaction to the stain.

3 days later, the wounds being uninfected and the animals apparently in good condition, Rat 2 was given a 15 minute exposure to the x-rays. 24 hours later both animals were killed, and the suspension of thymus cells in Locke's solution was counted in the usual way.

Rat 1 (control) gave 10 per cent of stained cells.

" 2 (x-rayed) " 23.5 " " " " "

The difference in the proportion of stained cells, however, does not fully indicate the severity of the injury. The cells of the x-rayed rat were in all stages of fragmentation, and accurate counting was impossible. It was observed that many of the chromatin particles, which made up the bulk of the suspended material, showed great resistance to the staining with trypan. This would indicate that not every form of cell injury is associated with increased permeability to the dyestuff.

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<sup>9</sup> Dr. M. J. Sittenfield kindly assisted me in this work.

We made a number of experiments to determine whether exposure of the serum to the x-ray in any way altered its protective power, and with one unexplained exception we obtained only negative results. It would appear therefore that the inhibitory property of the serum is not affected, at least during the first 6 hours. The possible existence of a latent period has not yet been tested.

*A Comparison of the Thymus Lymphocytes in Old and Young Animals.*

In view of the involutional changes which occur in the thymus, and less strikingly in the lymphatic tissues generally with advancing age, we compared with this technique the behavior of cells of old and of young animals. The increased resistance of the cells of young animals has been evident throughout the course of the work, but several experiments have been made to test this point specifically.

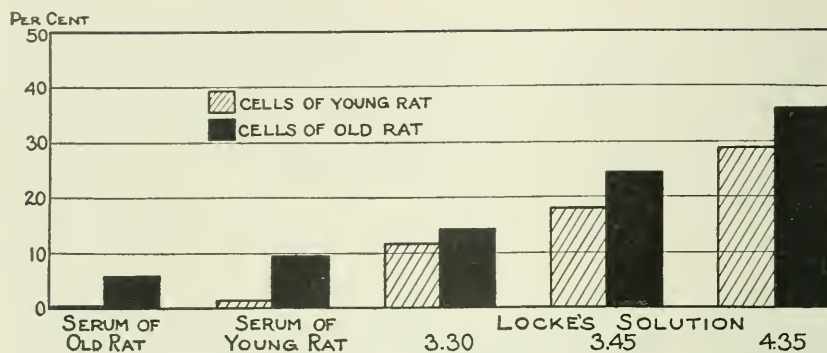
*Experiment 6.*—Suspensions were made from the thymus of a young rat and of a full grown adult animal. The cells were centrifuged and washed in two changes of Locke's solution to eliminate any possible action of the blood serum. The stainability of the cells was then compared in the usual way.

Time.....	Percentage of stained cells.		
	3.25 to 3.30	3.40 to 3.48	4.35 to 4.42
Young rat.....	11.1	18.2	28.7
Old rat.....	14.4	24.2	36.9

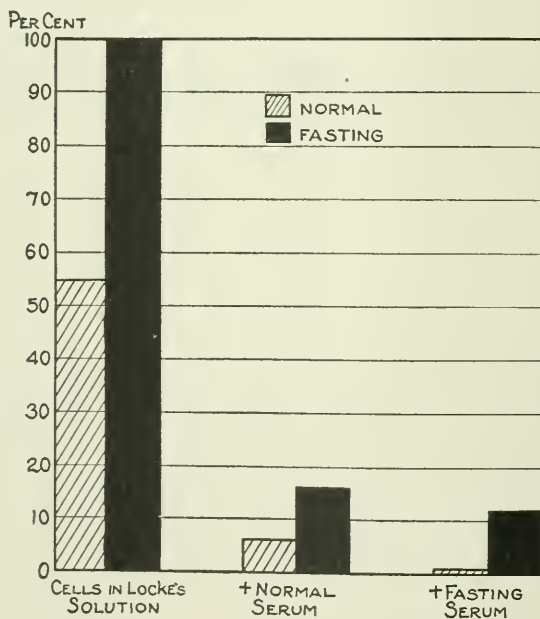
Several similar experiments gave identical results, so that there appears to be no doubt of the increased sensitiveness of the cells of the older animals to the unfavorable environment to which they are subjected under the conditions of the experiment. It will be interesting to extend this comparison to a study of the relative resistance to specific injuries of various kinds.

Fiore and Franchetti<sup>10</sup> in 1914 reported that they had produced premature involutional changes in rats by the injection of serum

<sup>10</sup> Fiore, G., and Franchetti, U., Su di una particolare proprietà del siero di sangue studiata in rapporto all'accrescimento dell'organismo è all'evoluzione del timo, *Atti Accad. med.-fis. fiorent.*, 1914, 87.



TEXT-FIG. 5. Experiment 6. The comparative stainability of thymus cells of young and of old rats.



TEXT-FIG. 6. Experiment 7. A comparison of the thymus cells and serum from normal and fasting rats.

from adult animals; and conversely, that the injection of "young" serum into old rats exercised a stimulating effect upon the growth of the thymus. This was contrary to our experience in as far as we had found the plasma of old animals a favorable medium for the growth of thymus tissue *in vitro*. In accord with other workers, we had found that the tissue of young animals showed a more vigorous growth than that of old.

However, the possible toxicity of the serum of old animals as compared with young ones was tested by subjecting young and old cells to the action of young and old serum. The comparative counts made with the usual technique do not indicate a toxic action on the part of the serum from old animals. When equal amounts of either young or old serum are added, there is always an increased stainability of the cells of the old animal. This is shown in Text-fig. 5.

*The Effect of Acute and Chronic Inanition upon the Stainability of the Thymus Lymphocytes.*

Perhaps the most striking feature in the pathology of the thymus is the atrophy brought about by acute starvation or prolonged inanition. This atrophy is due to the degeneration and partial disappearance of the small thymus cells, and leads, especially in the more protracted cases, to the most extreme reduction in the size and weight of the gland. The hunger involution has been repeatedly studied, both in the human subject, and experimentally by Jonson<sup>11</sup> and by Levin<sup>12</sup> in animals.

The following experiment, which has been performed several times with comparable results indicates that acute starvation brings about a greatly increased stainability of the thymus lymphocytes.

*Experiment 7.*—Rat 1, weighing 80 gm., was given water only for 3 days, during which period it lost 20 gm. in weight. It was then killed, serum was obtained and a suspension of thymus cells, twice centrifuged and washed, compared with a similar suspension from Rat 2, a control fed daily. Although this control rat

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<sup>11</sup> Jonson, A., Studien über Thymusinvolution; die akzidentelle Involution bei Hunger, *Arch. mikr. Anat.*, 1909, lxxiii, 390.

<sup>12</sup> Levin, S., Recherches expérimentales sur l'involution du thymus, Thèse de Paris, 1912, abstracted in *Zentr. exp. Med.*, 1912, ii, 262.



was severely infected with *Trypanosoma lewisi*, and in consequence ill nourished and anemic, it showed a lower count than the fasting rat (Text-fig. 6).

	Mixture.	Percentage of stained cells.
Rat 1 (fasting)	Locke's solution, 0.5 cc. Thymus cells, 5 gtt.	100
" 2 (control)	Locke's solution, 0.5 cc. Thymus cells, 5 gtt.	54.3

The unusually high count in the control is probably caused by the chronic malnutrition. The average proportion of stained cells in a healthy young rat, as seen by reference to previous experiments, is from 5 to 15 per cent.

The following data exemplify further the regularly noted difference between healthy and ill nourished, diseased animals.

*Experiment 8.*

Healthy rat.....	8.7 per cent.
Emaciated rat.....	27.0 " "

The question arose as to whether the serum from a fasting or badly nourished rat contained substances toxic for the thymus cells of a normal animal. It was found, contrary to this idea, that the sera of fasting or emaciated rats conferred a greater protection against staining than did those of the normal controls, possibly because of the greater concentration of the sera under these conditions.

An experiment designed to bring out similar differences in the protective action of sera from normal and marantic infants, upon the stainability of human tonsillar lymphocytes, gave no significant results. The two sera also conferred identical protection upon rat thymus cells.

*The Toxic Effect of Heterologous Sera.*

That the toxic effect of an alien serum may be manifested in a higher proportion of stained cells is shown in the following experiment, in which the action of rat serum is compared with that of two human sera, upon rat thymus cells.

*Experiment 9.*

Rat cells—rat serum, 2 gtt.....	1.4 per cent.
“ “ —human serum (A), 2 gtt.....	9.2 “ “
“ “ — “ “ (B), 2 “ .....	9.4 “ “

The toxicity of normal rabbit and guinea pig serum for rat thymus cells does not appear to be marked, although further studies are necessary. In one experiment the toxicity of horse serum was demonstrated, and also the fact that this toxicity, as judged by the increased percentage of stained cells, was not modified by heating to 56°C. for 15 minutes.

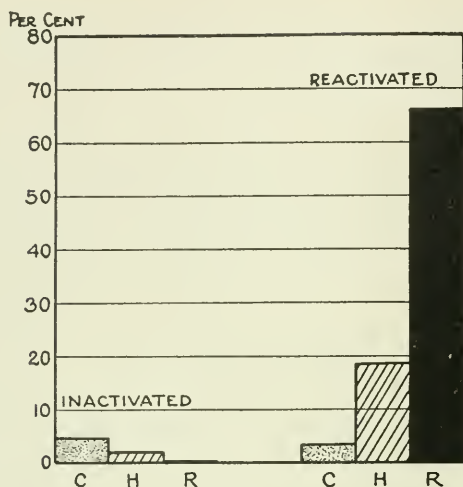
*Specific Cytotoxic Serum.*

A rabbit which had received four intravenous injections of 2 to 4 cc. of a thick suspension of rat thymus cells, has yielded a serum which is both agglutinative and cytotoxic. The agglutination is evident both micro- and macroscopically. The cytotoxic action is shown in the extreme morphological changes brought about in the cells in suspension (hydropic swelling of cytoplasm, pyknosis, karyolysis, etc.) and also sharply in the increased percentage of cells showing diffuse staining with trypan blue. Inactivation at 58–60°C. completely destroys the toxicity, and the addition of fresh guinea pig complement restores it. The agglutinin is not destroyed at this temperature.

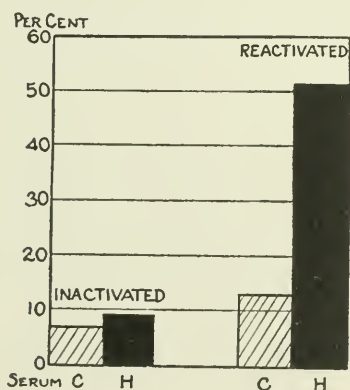
The serum is slightly hemolytic (1 : 10) for rat corpuscles and is also hemagglutinative. The hemolysin can be absorbed from inactivated serum by exposing it to washed thymus cells for 15 minutes at 37°C. On the other hand, exposure of the serum to red blood cells for the same period diminished, but did not wholly remove its thymotoxic action.

Text-figs. 7, 8, and 9 give some of the data upon which these statements are based.

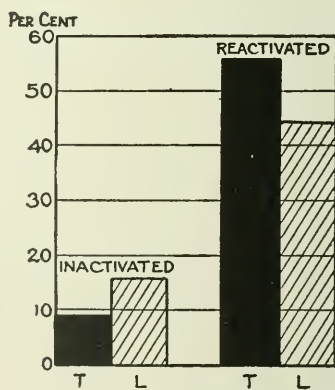
The serum of a rabbit immunized against human tonsil lymphocytes showed a slight toxicity for rat thymus cells as compared with the control normal rabbit serum. This suggests an organ-specific action upon lymphocytes, comparable with that established in the case of spermatoxin and lens precipitin. Further studies are necessary to determine this point. Against human tonsil lymphocytes,



TEXT-FIG. 7. Experiment 10. The effect of thymotoxic serum upon the stainability of rat thymus cells. *C*, control serum; *H*, serum of a rat immunized against human tonsil lymphocytes; *R*, serum of a rabbit immunized against rat thymus cells.



TEXT-FIG. 8. Experiment 11. The effect of lymphocytotoxic serum upon the stainability of human tonsil lymphocytes. *C*, control serum; *H*, serum of a rabbit immunized against human tonsil lymphocytes.



TEXT-FIG. 9. Experiment 12. The action of thymotoxic serum against lymphocytes from the thymus (*T*) and from a lymph gland (*L*).

the serum was highly toxic when reactivated with guinea pig complement. This is shown in Text-fig. 8.

Because of the discussion which has raged for many years over the question of the identity of the small thymus cells with the lymphocytes of other tissues, we undertook to test by this method the action of the thymotoxic serum upon the lymphocytes obtained from lymph glands.

Text-fig. 9 shows that both the agglutinative and cytotoxic actions are exerted equally upon lymphocytes of thymic and lymph gland origin. This seems to be strong evidence of their biological identity, more convincing perhaps than inferences based upon a common morphology.

#### CONCLUSIONS.

A simple method is presented by which, with the diffusion of trypan blue into the nucleus as a criterion of cell injury, it is possible to study quantitatively the effect of various agencies upon the small thymus cells and upon the tissue lymphocytes.

Preliminary studies with this method have led us to the following conclusions, which, however, unless otherwise stated, may be taken as applying only to the lymphocytes of the rat thymus.

1. The small thymus cells, when suspended in balanced phosphate solutions, show no distinct reaction to variations in hydrogen ion concentrations ranging between  $P_H$  7.0 and  $P_H$  7.8. Beyond  $P_H$  7.0 there is a sudden increase in the permeability of the cells to the dye; plasmolysis of the cells occurs when the alkalinity exceeds  $P_H$  8.0.

2. Heating to  $49^\circ$  or  $50^\circ C.$  is accompanied by a critical increase in the permeability of the cells to the dye.

3. The injury caused by lack of oxygen can be demonstrated by the increase in the number of stained cells.

4. The addition of serum to suspensions of thymus cells or tonsil lymphocytes greatly inhibits the diffusion of the trypan into the cells. The protection afforded is roughly proportionate to the amount of serum added.

Gelatin also exerts a marked protective influence; egg albumin affords a partial protection; starch and gum arabic are inert. Hemoglobin and cholesterol do not modify the stainability of the cells.

Arsenious sulfide in weak concentrations partially inhibits the diffusion of the dye. Colloidal iron is without effect, and is precipitated about the cells.

5. The toxicity of the photodynamic substance, hematoporphyrin, and of an impure chlorophyll solution in the presence of sunlight could be strikingly demonstrated by the greatly increased permeability of the cells to the stain.

6. Acute and chronic inanition produces an increased fragility of the cells. The protective power of the serum in acute starvation appears to be increased.

7. The small thymus cells of old animals are more readily injured than are those of young ones, as indicated by the increased proportion of stained cells.

8. The method has been applied to the demonstration of the action of cytotoxic immune sera for rat thymus cells and for human tonsil lymphocytes *in vitro*. Further experiments dealing with the question of specificity are in progress. The cytotoxins are inactivated by the addition of complement. Thermostabile cytagglutinins have also been produced.

I wish in conclusion to express my obligations to Miss Kate Brogan for her assistance in the technical part of the work.

## THE NORMAL FATE OF ERYTHROCYTES.

### I. THE FINDINGS IN HEALTHY ANIMALS.

BY PEYTON ROUS, M.D., AND OSWALD H. ROBERTSON, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 55 AND 56.

(Received for publication, January 22, 1917.)

It has long been recognized that in the healthy body a considerable proportion of the erythrocytes are broken down and replaced every day—exactly what proportion is not known. The bile pigments have been deemed an indicator of the hemoglobin destroyed. Calculations based upon their rate of formation would seem to show that blood destruction is very rapid, in man from one-tenth to one-fifteenth of all the corpuscles being lost and replaced in 24 hours.<sup>1</sup> But the recent work of Whipple,<sup>2</sup> who has proved that the bile pigments may have other sources than the blood, demonstrates a large possible error in such calculations. Perhaps the most certain evidence of blood destruction is to be found in the constant activity of the bone marrow in the production of new cells.

#### *The Phagocytosis of Red Cells.*

There exists an extensive but inconclusive literature on the normal method of destruction of the red cells. Pearce and Austin<sup>3</sup> say truly that only two facts regarding this method have been established: first, that certain large endothelial cells, located for the most part in the spleen, take up red corpuscles and destroy them; and, second, that blood pigment is sometimes present in the Kupffer cells of the liver,

<sup>1</sup> Calculated from figures given by Howell, W. H., *A Text-Book of Physiology for Medical Students and Physicians*, Philadelphia, 1917, 6th edition.

<sup>2</sup> Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

<sup>3</sup> Pearce, R. M., and Austin, J. H., *J. Exp. Med.*, 1912, xvi, 780.



indicating that they may play a part in the destruction. Some investigators hold that phagocytosis is of itself sufficient to account for blood destruction. In our work, this possibility has been the first considered.

Certain cells of the bone marrow and, exceptionally, of the lymph glands may ingest red corpuscles, but they are in general negligible as compared with the phagocytes in the spleen. Sections of the spleen, as ordinarily prepared, contain so much blood as to conceal the phagocytes in large part and render difficult an estimation of their number. For this reason, we have examined sections of spleens washed out with Locke's solution by way of the vessels, and have searched the centrifuged washings as well. In order to prevent all clotting in the latter, they have been mixed with an equal bulk of an isotonic, watery solution of sodium citrate (3.8 per cent). The spleens of the guinea pig, dog, cat, rabbit, and monkey have been thus examined. That of the guinea pig contains many phagocytes. Numbers of them come away in the washings, a fact easily understood when sections of the half washed organ are studied, for it is then found to contain many wide venules or sinuses in which the majority of the phagocytes lie free mingled with the red cells. The spleen of the rabbit contains similar venules. Phagocytes may be few or plentiful in this animal, depending on individual variation; and not infrequently a few appear in the washings. The numerous phagocytes of the dog spleen are all retained during perfusion in the densely reticulated organ. The bone marrow of this animal may show many cells containing red corpuscles and pigment. The *rhesus* monkey, like man, has few phagocytes normally. The cat shows a practically complete lack of such cells. Indeed, in several adult cats, of which we examined the spleen, bone marrow, lymph glands, and other organs, they were exceedingly rare. It is evident that, in this species, blood destruction must take place by some method other than phagocytosis.

There are wide differences in the size of the spleens of the common laboratory animals, which denote as clearly as the findings just described the dangers of generalization from the results in a single species. In the rabbit, the spleen is so small as to be, relatively speaking, almost vestigial. We have found that it weighs on the

average about 0.6 gm. in an 1,800 gm. animal, thus constituting about 0.033 per cent of the body weight. In the rat, it is proportionally some eight times as large, about 0.27 per cent of the body weight,<sup>4</sup> and in the dog and cat, it represents 0.18 to 0.28 per cent<sup>5</sup> of this weight. These differences would be noteworthy were they dependent merely on differing functional demands of one sort. But the findings as regards phagocytes in the spleens of different species show that at least one function of the organ is subject to wide variation.

Some authors have suggested that the phagocytosis of red cells is not a vital phenomenon, but is agonal or occurs after death. That such a view is erroneous can be shown by opening the splenic vein of an anesthetized guinea pig, which has been washed almost free of blood by means of Locke's solution, introduced through the jugular vein and allowed to escape from the carotid artery. Under these circumstances, if the spleen is gently massaged, many phagocytes filled with intact and fragmented red cells come away in the splenic washings. As in the case of the other animal species examined, the phagocytes are much larger than the mononuclear cells of the circulating blood which take up red cells under pathological conditions, being from 45 to 60  $\mu$  or more in diameter. We have attempted experiments on the conditions governing the normal ingestion of red cells, using for this purpose phagocytes washed from the rabbit spleen, but they failed to survive more than a few minutes *in vitro*, as proven by the staining of their nuclei with trypan blue.<sup>6</sup>

The number of corpuscles ingested in the large spleens of the rat and dog is so considerable that, *a priori*, in the absence of definite knowledge of the rate of blood destruction, one might suppose phagocytosis to be the means whereby, in these creatures, the destruction of erythrocytes is accomplished. So, too, in the guinea pig. But in the cat, phagocytosis does not come in for consideration; and in normal man, the monkey, and the rabbit, it cannot be held responsible for the disappearance of any important amount of blood.

<sup>4</sup> Jackson, C. M., *Am. J. Anat.*, 1915, xviii, 75.

<sup>5</sup> This statement is based on isolated instances from several authors to whose papers Professor Jackson has kindly referred us.

<sup>6</sup> Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443. Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 601.

*Extracellular Destruction.*

The search for extracellular blood destruction was now begun. Cats were employed for the first experiments. After positive findings had been obtained with them, the observations were extended to other species.

From what is known of the resistance of the red cells, it would seem unlikely that their destruction takes place while they are still in circulation. Tests with hemolytic and hypotonic solutions according to the current methods fail to disclose signs that any of them are in process of breaking down. Some are more frail than others, but the resistance of all is considerable. The washed corpuscles of man and the cat may be shaken in bulk for a considerable time without the destruction of any. All this is difficult to reconcile with the idea that the cells circulate while disintegrating. It would seem either that they are removed by some organ, or set of organs, as soon as they begin to break down, or else that they leave the circulation even before this happens. In either case, one might expect to find somewhere in the body of animals in which phagocytosis is negligible, as, for example, in cats, a locus of disintegrating corpuscles. We have thought to find it by a search of the washings from organs perfused until entirely blood-free, combined with an examination of the washed organs themselves. Such a search presupposes a perfusion fluid in which the corpuscles will not be injured, and a method whereby those that are undergoing the normal disintegration may be collected from out the great mass of intact cells. The first of these requirements is fulfilled by the use of Locke's solution<sup>7</sup> containing a small amount of gelatin, and the second by differential centrifugation of the washings. When freed of serum and placed in normal salt solution, or Locke's or Ringer's solution, the erythrocytes of many species, especially those of the dog and of some rabbits, begin within a few hours to hemolyze. This, as one of us has shown, is the result of mechanical injury, entailed in even the most careful handling. It can be avoided by adding to the solution 0.125 to 0.25 per cent of gelatin.<sup>8</sup> In such a

<sup>7</sup> Locke's modification of Ringer's solution, but without sugar.

<sup>8</sup> Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

fluid, delicate corpuscles remain intact, although pipetted or even shaken. The present work has constituted itself a severe test of the efficacy of the protection afforded by the gelatin. For the cell shadows that are the product of mechanical injury are easily recognizable, and by the differential centrifugation we have employed, they are brought into the preparations examined. Rarely has one been observed. Indeed, their consistent absence from the specimens obtained on perfusion and from teased preparations of the organs perfused renders it certain that normal blood destruction does not entail the formation of shadows.

We reasoned that disintegrating red cells would probably be lighter than sound ones because of some loss in substance or content. If this were true, they should come down last when the blood is slowly centrifuged in dilute suspension. It proved to be the case in preliminary tests with suspensions of the damaged cells of animals injected intravenously with a specific hemolysin.

#### *Method.*

A specimen of the animal's blood was taken by direct cardiac aspiration into a mixture of perfusion fluid and citrate solution, and then, under ether, the viscera were excised one at a time, after the vessels had been temporarily tied or clamped off, and immediately flushed out through the artery with Locke's solution containing 0.25 per cent of gelatin. A large gravity bottle was employed for the perfusion. Before it was begun, each organ was rinsed of surface blood, and during the process it lay in a porcelain dish into which the washings escaped by way of the natural venous channels. The perfusion pressure was ordinarily that of a column of the fluid some 120 cm. high. The washings which came last, and which were thought to represent, not so much the circulating as the residual blood, were caught separately and examined with special care. The kidneys, intestines, liver, and lungs were easily washed out. In order to wash the red bone marrow, a femur was excised, its surface flushed with the perfusion fluid, the bone was chipped away, and the marrow washed directly by means of hollow needles thrust repeatedly into it. In some cases, a limb of the animal was perfused *in situ*. Examination showed that the marrow then failed to wash out. The spleen and bone marrow were the only organs from which the blood failed to come away completely. In them a considerable trace always remained.

To prevent clotting the washings were caught in an equal bulk of isotonic, watery, sodium citrate solution (3.8 per cent). In the later work a mixture of equal parts of the citrate solution and gelatin-Locke's solution was used for the perfusion. 0.25 per cent of gelatin in the wash fluid was found to ensure protection in this ultimate mixture.



Portions of the washing were now slowly centrifuged in tall test-tubes and when the mass of red cells had come down, the shimmering, faintly pink, supernatant fluid was removed to another tube and sedimented at high speed. The slight sediment was suspended in a trace of fluid and examined microscopically. It consisted for the most part of platelets, red cells, and fragments of red cells.

The specific gravity of the wash fluid was such that no distinct leukocytic pellicle was formed.

#### *Bodies Like Degenerated Red Cells.*

Examinations of the organs of cats, rabbits, monkeys, and dogs, carried out according to this method, disclosed the presence of peculiar bodies in the washings from the spleen, bone marrow, and especially the liver, which yielded them in great number. They had the appearance of red cells and cell fragments which had lost their hemoglobin, but, unlike shadows, had retained something of their other substance, so that they were still refractile, though never markedly so (Fig. 1). They were not found in the washings from lung, limbs, or intestines. The kidney yielded a few of them. On centrifugation, they came down with the platelets, and when in great numbers, as in the liver washings, they tended on standing to collect into clumps (Fig. 1), which, however, were easily broken up with the pipette. They were spherical, oblong, or sausage-shaped, of all sizes up to that of the red cell or slightly larger, and of many degrees of refractility, some, especially the smaller, being difficult to perceive. The substance of the best preserved had often a greenish yellow cast, and what appeared to be transition forms from red corpuscles were easily found. When placed in blood serum, the appearance of the bodies did not change. Methylene blue and cresyl blue brought out in them a reticulum somewhat more delicate, as a rule, than that of reticulated red cells, but often indistinguishable from it (Fig. 2). Scharlach R and Lugol's solution failed to stain them.

Like red cells, the bodies were laked by water and by bile, acetic acid, saponin in high dilutions (1: 10,000), and by serum hemolysins when complement was present. In all these instances, shadows were left behind similar, except for the variation in size, to those of red cells. The bodies laked somewhat more easily than red cells, as was seen in preparations containing both. On standing in the ice box in salt solution, they became shadows after some hours or days. They

were crenated by hypertonic salt solutions, fixed by formalin and Cupp's fluid (Fig. 3), and agglutinated by specific agglutinins for the red cells. Elements morphologically indistinguishable from them were separated out by differential centrifugation from the blood of rabbits that had become anemic as the result of repeated injections of a serum hemolysin. We repeatedly recovered elements resembling them from the blood of patients with severe pernicious anemia, and from one case of congenital hemolytic jaundice. Other observers<sup>9</sup> have also found them in these conditions. They were regularly found in the circulation of rabbits engaged in breaking down a plethora of blood transfused to them from other rabbits. Furthermore, elements resembling them were observed within the endothelial phagocytes of rabbits injected intravenously with an hemolysin.

Despite all these characters which would seem to identify the bodies with disintegrating red corpuscles, they have another origin, being the products of injured cells of the fixed tissues. This can be shown by washing out at 5 minute intervals the liver of a rabbit, dog, or cat while it is still *in situ*. If ligation of the hepatic artery and the introduction of cannulas into the portal vein and the hepatic end of the inferior vena cava are carried out so rapidly that perfusion supervenes on normal circulation after the lapse of only 1 or 2 minutes, no bodies, or at most only a few will come away in the first washings which free the liver of blood. If there has been delay, the bodies will be found. Always they occur in great number in the washings obtained by perfusion after 5, 10, and 15 minutes. The result is the same whether the fluid used is a salt solution or defibrinated blood. In teased specimens of the liver and spleen, the cells of the parenchyma not infrequently may be seen to give off translucent globules, some of which when free constitute the bodies. Splenic phagocytes containing red corpuscles have been observed to give off "bodies" that are brightly tinted with hemoglobin.

If this is the origin of the bodies, how do they come to circulate in the blood of animals and human beings, the subjects of hemolytic processes? Those seen under such circumstances appear to have

<sup>9</sup> Lee, R. I., Minot, G. R., and Vincent, B., *J. Am. Med. Assn.*, 1916, lxvii, 719.



another source. They are probably reticulated red corpuscles, free of hemoglobin. Though morphologically such elements are characteristic "bodies," their reticulum as demonstrated with cresyl blue is moored in place, so to speak, as is true of reticulated cells in general, whereas that of the bodies usually draws together toward one side into a heavily pigmented skein or ball, some minutes after staining (Fig. 2).

The bodies differ in many ways from Albrecht's myelin, which is a relatively late postmortem product. A discussion of them at such length has seemed warranted because elsewhere we<sup>10</sup> have erroneously reported upon them as disintegrating red cells, and because they do indeed possess many characters which have been regarded as peculiar to erythrocytes. Their study should prove profitable to those interested in the constitution of the red cell.

In the subsequent work, each organ was washed out with all possible speed after the circulation had been interrupted. Often this was done with the organ *in situ*. As a result, the "bodies" have seldom been encountered.

### *Disintegration by Fragmentation.*

The method just described has made possible a close search of the organs, one by one, for disintegrating red cells. Nowhere have hemolyzing cells been found; and the consistent absence from the washings of shadows, as already mentioned, is additional evidence that hemolysis in the gross sense does not occur. Instead, another and unsuspected method of blood destruction has been found; namely, disintegration of the cells, while they are still circulating, by fragmentation without loss of hemoglobin. It occurs in the cat, rabbit, and dog, all the animal species thus far examined.

If 2.5 cc. of blood is taken direct from the heart of a normal animal into 15 cc. of gelatin-Locke's-citrate and submitted to differential centrifugation, microcytes and poikilocytes similar to those of anemic bloods are regularly found, often in considerable numbers, among the platelets and other elements that are last to be thrown down. All stages in their derivation from red cells of normal size can be observed.

<sup>10</sup> Rous, P., and Robertson, O. H., Association of American Physicians, 1916.

Among the few of these latter that are associated with the small forms in the ultimate sediment, some are fragmenting, as shown by deep constrictions in their protoplasm or by their possession of slender processes from which small, hemoglobin-containing bits are separating off. The cells first thrown down by the centrifuge, that is to say the great bulk of the red cells, fail to show any such changes.

The division of red cells into microcytes and poikilocytes—schizocytes, as Ehrlich called them and as we shall for convenience refer to them—can be brought about *in vitro* by the pressure of a cover-glass, or by gently heating the fresh preparation (Schultze, Rollet, Ranvier<sup>11</sup>). Such forms may also appear in the blood during caffeine poisoning and after prolonged anesthesia with chloroform.<sup>11</sup> The ease with which they are produced shows the need for careful controls to our observations. These have been carried out. Repeated manipulation of the diluted blood according to the method used in our work does not increase the number of fragmenting forms. They are found when the blood has been taken into oxalated salt solution instead of gelatin-Locke's-citrate. They occur in the blood from anesthetized animals. And, finally, they are present in undifferentiated fresh blood, though they are in general so few that it is easy to see why they have not been recognized as normally present there. They may easily be found in fresh rabbit blood. In looking for them care should be taken to use thick films of the blood, since in thin ones the cells tend to catch on the glass surfaces by which they are compressed and to be pulled in two as the drop spreads, thus producing an artificial fragmentation. This is avoided when the blood is examined in a thick layer or caught directly in a little gelatin-Locke's-citrate and then observed. Under both these circumstances, the number of fragmentation forms is usually small, and indeed some rabbit bloods fail to show them except on long search. In most bloods, though, they are regularly found, one to twenty being noted as a rule in a 5 minutes' search. In the individual the number is remarkably constant from day to day. It may be considerably increased when there is the least anemia.

In the washings from the organs, as obtained by our method,

<sup>11</sup> Cited in Krehl, L., and Marchand, F., *Handbuch der allgemeinen Pathologie*, Leipzig, 1908, i.

much normal blood is always present, and in consequence schizocytes are always found. It has been necessary to control this factor in the search for accumulations of disintegrating cells. Our practice has been to make in perfusion fluid suspensions of the animal's heart's blood of approximately the same hemoglobin content as each of the organ washings, and to differentiate them in the centrifuge with the latter. Such dilute specimens of the circulating blood yield few schizocytes, and any marked preponderance of them in the washings can with good reason be attributed to the organ's special content.

The results of our search of the organs can be stated briefly. The only evidence encountered of extracellular blood destruction lay in the presence, in the spleen especially, of accumulations of microcytes and poikilocytes such as had been met with in the circulating blood. The spleens of the dog, rabbit, and cat regularly contained numbers of these small forms. They often failed in large part to wash out, being then discoverable in the tissue teased after perfusion. Systematic observations in rabbits have shown that there may be small collections of them elsewhere, most frequently in the kidneys, but these are inconstant and always insignificant as compared with the splenic content, even when the difference in size of the organs is taken into account. The red bone marrow (femur and rib) is notably free of the forms. It is plain that they are not put forth as such by the marrow. Confirmatory evidence on this point is presented in our second paper.

The normal spleen may retain sufficient schizocytes after washing to give the organ a brownish red color, as in the case of many rabbits and cats, or this color may be due in greater part to phagocytized red cells, as in other rabbits and in dogs. In some healthy animals, certainly, the accumulation of schizocytes is not great. Some cat spleens can be washed almost white, and the washings in these cases may contain only moderate numbers of the fragmentation forms. Their number is moderate, too, in the large spleen of the dog. But they are always present and are much more frequent than in the circulating blood. The difficulty with which they are dislodged from the spleen, even on repeated perfusion, is striking. The first

washings in which most of the blood comes away contain but few of them.

A number of control tests have been made which prove that the splenic schizocytes are not artefacts. They are regularly found when the fresh organ is teased in autogenous blood serum. The teasing itself does not cause them, for other organs containing the same amount of blood and similarly teased fail to yield them. They have no relation to the use of ether as an anesthetic. The possibility that they are fragments of corpuscles extruded by phagocytes in animals possessing such cells has been ruled out by means of a differential stain. In fresh preparations in isotonic salt solution, to which a little cresyl blue has been added, the fragments of red corpuscles resulting from intraphagocytic digestion become a deep, opaque blue-black, whereas those produced by extracellular fragmentation fail to stain at all. The specificity of the dye is, in this regard, striking. Red cells when first phagocyted fail to stain. Later they stain copper-green or intensely blue. As breaking down proceeds, the affinity for the stain grows, and the intracellular fragments of red cells stain almost black in solutions of cresyl blue so weak as scarcely to affect other elements. In teased specimens, these blue-black fragments are not infrequently found lying free, a sharp contrast to the non-staining fragments (schizocytes) that result from extracellular destruction.

### *The Schizocytes.*

The shape of the poikilocytes and microcytes found in the peripheral blood and the spleen is to some extent peculiar to the animal species. In the cat spherical or oblong forms predominate (Fig. 4); in the dog, short, blunt rods exist as well; in man, in addition to these, shapes like small asymmetrical starfish are observed; while in the rabbit, there is the greatest diversity of forms—spheres, capsules, shapes like drumsticks, rods, dumb-bells, pears, balls with strings to them, and even short, thick threads brightly tinted with hemoglobin (Fig. 5). If anything, the schizocytes are of a more intense orange hue than ordinary corpuscles. Many very small forms are seen. From the larger forms, fragments so minute as to be at the limit of microscopic visibility can be seen to break off. The



ultimate fate of all would seem to be division and redivision into a fine dust.

Red cells of approximately normal size, from which fragments are in process of separation, are fairly common in the spleens of all the species we have examined. As already stated, they are present in the ultimate sediment obtained on differential centrifugation of the circulating blood. A form always to be found in the rabbit spleen, and sometimes recovered from the blood by differential centrifugation, consists of two deeply tinted portions of corpuscle held together by a median zone of protoplasm so flattened anteroposteriorly as to appear devoid of hemoglobin (Fig. 6), except when viewed from the side. When so viewed this form has a dumb-bell shape, and the connecting zone is seen to be well provided with pigment. In the end the zone gives way, and the cell portions become separate microcytes. We have never found any indication of the decolorization of microcytes in the spleen. In the cat spleen there may sometimes be encountered a few pale and exceedingly thin corpuscles, still tinted with hemoglobin, which look as though they were dissolving away much as discs of sugar dissolve in water.

#### SUMMARY.

The phagocytosis of red corpuscles, while frequent in the normal dog, rat, and guinea pig, is slight in man, the *rhesus* monkey, and many rabbits. In cats it is always negligible in amount and frequently absent. Phagocytosis will not suffice as a general explanation of normal blood destruction.

When the liver, spleen, and bone marrow of the cat, dog, rabbit, or monkey are slowly perfused with defibrinated blood or Locke's solution, bodies are given off into the fluid which have the appearance of red corpuscles that have lost their hemoglobin but retained the rest of their cell substance. These bodies possess many of the properties supposedly distinctive of red corpuscles. They are the product of disordered parenchymal cells.

By a special method, it has proved possible to search the body, organ by organ, and the circulating blood also, for disintegrating red corpuscles. Shadows of red cells are not present anywhere, nor are hemolyzing red cells found. A hemolytic process, in the

ordinary sense of the term, can scarcely play an important part in normal blood destruction. Instead, it is certain that some red corpuscles, at least, are destroyed in another way; namely, by fragmentation. Normal blood regularly contains small numbers of fragmentation forms—microcytes and poikilocytes—and accumulations of them are regularly present in the spleen, but are found only inconstantly in the other organs. The fragments are in evident process of further subdivision. They occur not only in species in which phagocytosis as a means of cell destruction is negligible (cats), but also in animals in which it is an important process (dogs, some rabbits).

The method of study that we have employed is well suited to disclose how the blood is destroyed. The importance of cell fragmentation in this connection is indicated by our failure to find any other means of destruction, save only the phagocytosis already known. Further facts indicating the importance of fragmentation are presented in our second paper, where a general discussion will also be found.

#### EXPLANATION OF PLATES.

##### PLATE 55.

FIG. 1. "Bodies" from the liver washings of a dog. Five ordinary red cells and a leukocyte are also to be seen. The arrows point to the bodies, which are clumped as the result of centrifugation. Fresh preparation.

FIG. 2. "Bodies" stained with cresyl blue. A, immediately after staining; diffuse reticulum. B, later effect; the reticulum has massed to one side. From the liver washings of a dog.

##### PLATE 56.

FIG. 3. "Bodies" fixed with Cupp's fluid (a formalin-potassium-bichromate-acetic-acid mixture). A, bodies; B, red cells. From the liver washings of a monkey.

FIG. 4. Microcytes and poikilocytes collected by differential centrifugation from the blood of a normal cat. Wright's stain. The upper arrow points to a round microcyte, and the lower to a poorly focused oblong form.

FIG. 5. Microcytes and poikilocytes collected from the blood of a normal rabbit. The dark dots are platelets. The arrows point to the schizocytes of which the diversity of form is well shown. Wright's stain.

FIG. 6. Fragmenting red corpuscles from the spleen of a rabbit. Wright's stain. There is no real loss of hemoglobin from the pale portions of the cells, but here the layer of protoplasm is very thin.





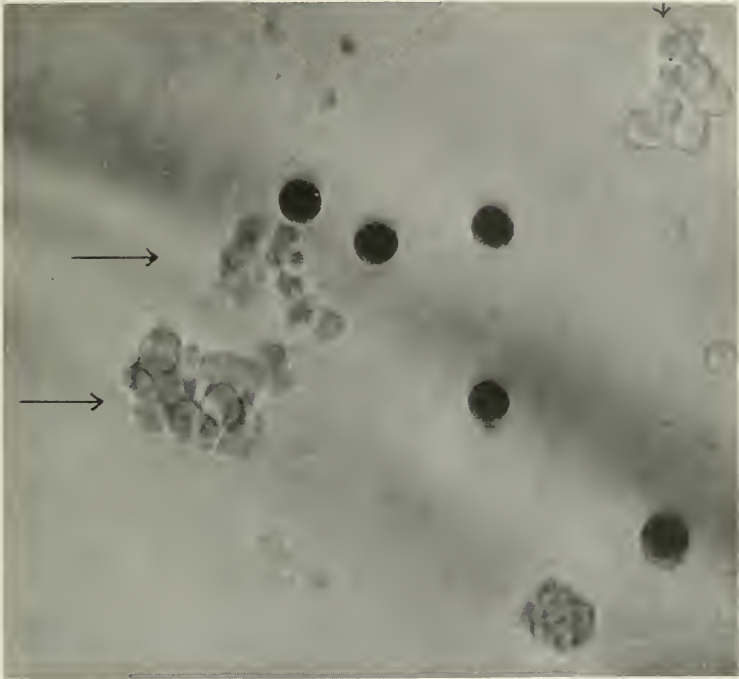


FIG. 1.

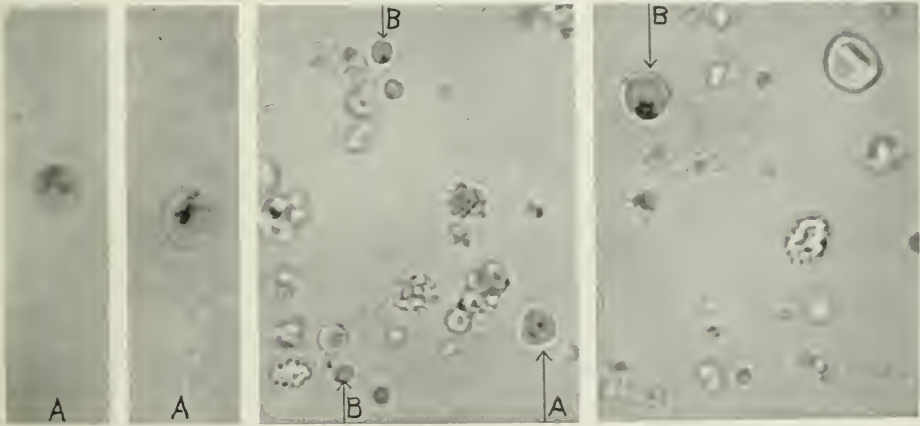


FIG. 2.

(Rous and Robertson: Normal Fate of Erythrocytes. I.)



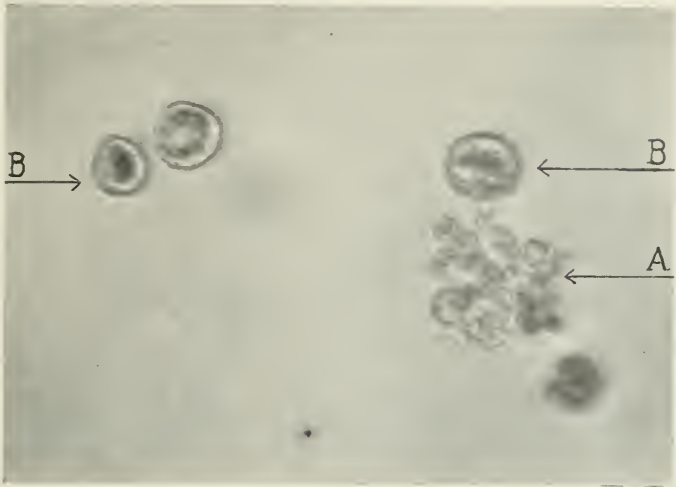


FIG. 3.

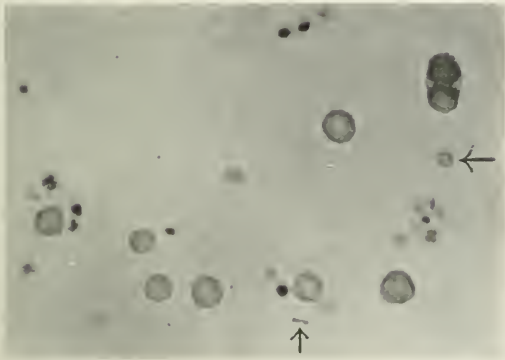


FIG. 4.

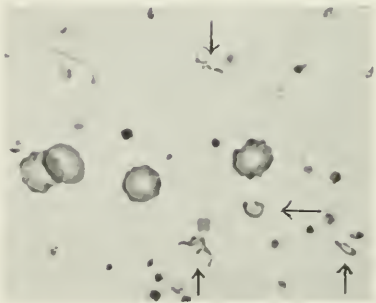


FIG. 5.

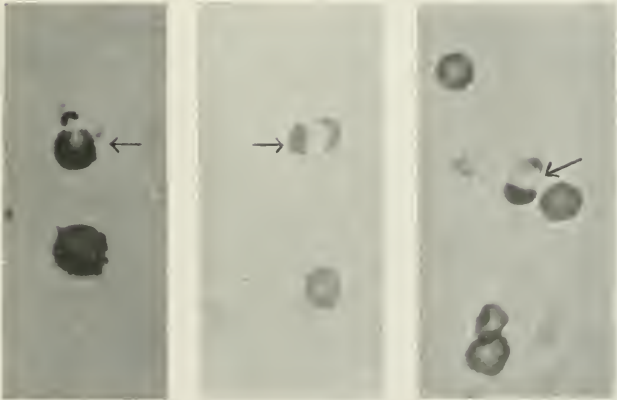


FIG. 6.

(Rous and Robertson: Normal Fate of Erythrocytes. 1.)



## THE NORMAL FATE OF ERYTHROCYTES.

### II. BLOOD DESTRUCTION IN PLETHORIC ANIMALS AND IN ANIMALS WITH A SIMPLE ANEMIA.

BY OSWALD H. ROBERTSON, M.D., AND PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The findings presented in the preceding paper show clearly that phagocytosis can account for red cell destruction only in certain species, and that whatever the extracellular method of this destruction may be, it does not entail the formation of shadows, such as result from hemolysis. The evidence is against a direct hemolytic action in the spleen. The constant presence in this organ of an accumulation of poikilocytes which are subdividing, and of microcytes, and the presence of these elements in the circulating blood indicate that the red cells disappear, in part at least, by fragmentation. In a further study of methods of blood destruction, we have examined rabbits rendered plethoric by repeated direct transfusion. Such animals soon acquire the ability to dispose of large quantities of blood. This goes on in the absence of demonstrable agglutinins or hemolysins, and, according to Boycott and Douglas,<sup>1</sup> represents an intensification of the normal process of destruction, though what that is the authors cited could not discover. They state that the spleen is the only organ in which changes are regularly met with. It is enlarged, and phagocytes containing red cells are more numerous than usual.

#### *Method.*

Rabbits were used. From three to six compatible donors were selected for each recipient. The donors were employed in rotation, and each recipient was given nearly every day 10 cc. of whole blood from one of them. The blood was

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<sup>1</sup> Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909-10, xiv, 294.



obtained by cardiac aspiration into a syringe containing 1 cc. of 1 per cent sodium citrate in 0.9 per cent salt solution. It was injected directly into an ear vein of the recipient. Clotting was not met with. The small amount of citrate—0.01 gm. at each injection—caused no symptoms or lesions.<sup>2</sup> The transfusions were continued for several weeks, during which time the animals remained well and frequently gained in weight.

The rabbits were found to differ much in their ability to dispose of the excess blood. In some, it was difficult to increase the hemoglobin value even by daily injections; in others, it began at once to rise, and soon reached a high figure. Since the total blood volume from day to day was not known, it is impossible to say how much these differences are attributable to variations in the plasma. As a rule, when the animal had been transfused for some time, and the hemoglobin had increased from 80 or 90 per cent Sahli to about 150 per cent, it failed to go higher, but instead tended to drop despite the daily addition of blood. Yet very often in such animals, no demonstrable agglutinins had developed. These animals were judged suitable for our purpose. The circulating blood was examined for disintegrating red cells, the animal was etherized, and the organs were washed out one by one according to the methods outlined in our first paper. The washings were submitted to differential centrifugation, and portions of the organs were teased and examined.

The circulating blood of many of the plethoric animals showed microcytes and poikilocytes—schizocytes—in far greater numbers than did that of normal controls; but no other signs of blood destruction were seen in it. Examination of the donor rabbits showed that the increase in microcytes and poikilocytes was in general traceable to them. The removal from these animals of 10 cc. of blood every 4 or 5 days was often sufficient to cause a marked increase in the number of schizocytes in circulation, whence they were transferred to the plethoric individuals. When care was taken to use donors with few circulating schizocytes, it was found that the increased blood destruction in the plethoric rabbits was unaccompanied by an increase in these forms in the blood.

There was regularly found a notable increase in the number of schizocytes in some of the organs under circumstances which precluded an introduction of them with the donor's blood. For example, in one animal repeatedly transfused during 14 days, and always with a blood in which schizocytes were rare, the spleen was found to be packed with these forms. It was a large, brown-purple organ, from

<sup>2</sup> In control animals injected with sodium citrate alone, normal findings were obtained.

which several times as much blood as normal was obtained on perfusion. After washing, it was smaller than before, but still enlarged, weighing 1.1 gm. as compared with 0.6 gm. for the average normal individual of the same size. In the teased specimen phagocytosis was only slightly increased, but there were great numbers of microcytes, poikilocytes, and the peculiar dumb-bell form of fragmenting red cell encountered not infrequently in normal animals.<sup>3</sup> In fact, nearly all the considerable quantity of blood which failed to wash from the spleen consisted of these forms. In the washings they were far more frequent than is ever the case in normal blood. No agglutinated masses or shadows were observed.

This is a typical finding. In some spleens, small extracellular aggregates of amorphous brown pigment were frequent. Sometimes phagocytosis was increased, but often it was only normal. The one unfailing and characteristic feature was the great accumulation of schizocytes.

In the other organs, with the exception of the red bone marrow, the accumulation of schizocytes was inconstant and always negligible. The fact has already been brought out that the normal marrow contains only such schizocytes as happen to be present in the blood coursing through it. In the plethoric marrow, on the contrary, they are met in considerable numbers. Occasionally the number of phagocytes containing red cells is somewhat increased. We have utilized the peculiar affinity of the cresyl blue stain for intracellular red cells and their fragments<sup>3</sup> to determine whether the free schizocytes in marrow and spleen are the product of intraphagocytic digestion. This is not the case.

Despite careful search, no other methods of blood destruction were observed. The findings strongly support the view that the disappearance of red cells in plethoric rabbits takes place in large part by fragmentation, if not almost wholly.

#### *Blood Destruction in Animals Anemic from Hemorrhage.*

Microcytes and poikilocytes are frequent in the blood of animals rendered anemic by hemorrhage. The conception that these forms

<sup>3</sup>Described in the preceding paper.

are the result of blood destruction finds here an apparent contradiction; for in simple anemia one would expect a conservation of blood rather than increased destruction.

We have examined numerous anemic rabbits by perfusion and differentiation of the washings in the centrifuge, according to the method already described.<sup>3</sup> To render the animals anemic they were bled on successive days by cardiac aspiration, 25 cc. being the usual amount of blood removed. After four or five bleedings, when the hemoglobin had dropped to 30 to 20 per cent, no more blood was taken. 3 or 4 days after the last bleeding, films of the blood showed that repair was in progress. There were many reticulated corpuscles, numerous large pale ones, and, in addition, marked polychromasia and numerous microcytes and poikilocytes. It was at this stage that the animal was etherized and the organs examined.

The findings may be briefly summarized. There was a striking increase in the spleen's content in microcytes and poikilocytes. Much of its residual blood consisted of these forms, and, in several instances, the organ was somewhat enlarged from their accumulation. Phagocytosis was not increased. In some of the other organs, especially in the kidney, small collections of microcytes and poikilocytes were inconstantly present. It was noteworthy that the washings from the marrow never yielded more of them than did the control specimen of circulating blood, while in the marrow tissue teased after washing, there were practically none. The point had special attention because of the widely held view that these forms originate as such from the blood-forming tissue. Our findings show that this is not the case, and that the microcytes and poikilocytes of anemia from hemorrhage are true fragmentation forms, deserving of the name of schizocytes given to them by Ehrlich.

Several facts were observed which indicate that the schizocytes of anemia are derived for the most part not from the fragmentation of cells remaining from the pre-anemic period, but from the breaking down of cells formed by the bone marrow to make up the lack in blood. Most of the schizocytes of the anemic rabbits partook of the pallor of the new-formed corpuscles. In the centrifuge, they were readily separated from the mass of cells but were themselves completely sedimented only with difficulty; and the last

to come down from suspension were practically colorless. Large numbers of the schizocytes were reticulated like the new corpuscles circulating with them. In two instances in which counts were made, 56 and 25 per cent of the schizocytes were reticulated, as compared respectively with 30 and 24 per cent of the corpuscles, a relation readily understood when it is considered that one reticulated corpuscle might give rise to several reticulated schizocytes. In the normal rabbit, as is well known, 2 per cent of the corpuscles or less show reticulation, while according to our experience, none of the schizocytes are reticulated. In the anemic rabbit, reticulated schizocytes appear almost immediately after any great increase in reticulated corpuscles. The conclusion seems warranted that the schizocytes of the anemia result from the fragmentation of new-formed, abnormal red cells. Some of the latter must begin to break in pieces almost as soon as they are put forth by the marrow. From the study of a fresh preparation of anemic blood, some idea can be had of the ease with which this may occur. As the blood drop spreads under pressure of the cover-glass, it will be observed that the well colored, normal looking red cells constitute stable objects as compared with the pale, large cells resulting from the anemia. Often one of the latter will be swept through a narrow cranny between two normal cells. The contour of these latter does not change in the least, whereas the pale cell is greatly compressed, and it flows through the aperture as if fluid. It is easy to see how such cells may be pulled to pieces in the circulation.

Altogether, the findings in anemic animals indicate that here the destruction of red cells by fragmentation goes on actively. Large numbers of the fragments accumulate in the spleen. Many, though, circulate for a greater or less time. This is not the case with the schizocytes of normal and plethoric animals, which are rapidly taken out of the blood.

#### DISCUSSION.

Ehrlich long ago stated<sup>4</sup> that the microcytes and poikilocytes of anemia result from fragmentation of the circulating cells. He

<sup>4</sup> Ehrlich, P., *Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes*, Berlin, 1891, 99.



based his view on the fragmentation *in vitro* of red cells under the action of various physical and chemical agents. His conception is still far from having gained general acceptance, many hematologists holding the view that microcytes and poikilocytes are elaborated as such in the marrow.<sup>5</sup> Our findings show definitely that they arise in the circulation. Ehrlich thought that their appearance during an anemia was purposeful, since it increased the blood surface, and, as a corollary to this opinion, he held that they circulated for some time. Two of our facts point against the idea of a purposeful division. The cells which break down are those least suited to combat the anemia, namely, those poor in hemoglobin; and the fragments tend to accumulate in the spleen, whence they cannot easily be flushed out. Cell fragmentation in an anemic organism would seem to be, not the result of a compensatory arrangement, but an incident in a vicious circle. Because of the anemia, the bone marrow puts forth cells that are unable to withstand the exigencies of circulation. These soon fragment and the work of the marrow must be done again. The marrow then must not only make up an initial blood lack, but while so doing must repair constant fresh losses due to the poor quality of the cells it puts forth. Or, in other words, it must take one step down for every two steps up. That there is no increase in the output of bile pigments in anemic animals may be due to a special conservation of hemoglobin, or to the relative paucity of this substance in the broken down cells, or to a diminution in the breaking down of such normal red cells as are present, or to extraneous factors such as Whipple has shown can influence the pigment output.

The view that red cell destruction normally takes place for the most part by fragmentation finds striking support in the findings in both anemic and plethoric animals. In the former, the red cells are less hardy than usual. Fragmentation is the result. In the latter, a greater blood destruction than usual must be accomplished. Again fragmentation is increased. Furthermore, in our plethoric animals, which would seem especially suited to the investi-

<sup>5</sup> For the latest statement of this view see Barker, L. F., *The Clinical Diagnosis of Internal Diseases*, New York, 1916, iii, 25.

gation, no other method of destruction than fragmentation, saving only the phagocytosis already known to exist, has been encountered. Phagocytosis is often increased in these animals, but not always. In some cases, fragmentation is practically the only process of destruction found.

The accumulation of schizocytes in the spleen is striking, both in anemic and plethoric animals. That this organ has some important function in connection with such elements cannot be doubted; and the findings in plethoric animals suggest that the bone marrow may share the function if blood destruction is great. When human beings are severely burnt, many red cells break into hemoglobin-containing fragments. According to good authority,<sup>6</sup> these collect in the spleen so rapidly that within a few minutes practically all are removed from the blood. Not improbably the blood of normal and plethoric animals is kept free of fragments in much the same way. They are not taken so completely from the blood of anemic animals. Perhaps a change in the protoplasm, such as may be thought to accompany normal aging of the cell, is necessary for this. But even in anemia, microcytes and poikilocytes accumulate in the spleen to a noteworthy extent.

The schizocytes are undoubtedly reduced at last to a fine hemoglobin-containing dust. How this is disposed of, whether in a special organ, like the spleen, or by dissolution in the blood is not evident. But it must be disposed of rapidly, or the blood of plethoric animals would be rich in it. The hemoglobin, wherever and however given off, will of course be quickly utilized by the liver.

Taken together, the facts afford a simple and rational explanation of the normal method of blood destruction, and indicate why the problem has presented difficulties. A constant rapid fragmentation of the effete red cells, one by one, while still circulating, and a prompt utilization of the products of destruction will readily account for the high general standard of cell resistance that has puzzled observers. Whether, indeed, as the red cells fragment, their resistance lessens, as determined by the ordinary tests, remains to be determined. The

<sup>6</sup> Krehl, L., and Marchand, F., *Handbuch der allgemeinen Pathologie*, Leipsic, 1908, i.



fragmentation does not involve a loss of hemoglobin, and *a priori* there is no reason why it should be accompanied by a decreased resistance to hemolysins or hypotonic fluids, the ordinary test-agents.

Meltzer<sup>7</sup> has suggested that the red cells are normally destroyed through mechanical wear and tear, and has studied in this connection their fate when shaken. But cells shaken *in vitro* are not destroyed according to the method we have encountered in the body. Only a few of the shaken cells break into microcytes and poikilocytes. The vast majority are reduced to shadows. Shaking is obviously a different process from the perpetual sieving and squeezing of the cells that go on in the finer capillaries. Yet it is possible that the resistance of the corpuscles to shaking may after all give some indication of their tendency to fragment. Experiments to determine the point are now under way.

#### GENERAL CONCLUSIONS.

1. The increased destruction of red cells in animals rendered plethoric by transfusion takes place predominantly by a fragmentation of the corpuscles without loss of hemoglobin.

2. The microcytes and poikilocytes observed in animals with a severe anemia due to hemorrhage are not put forth as such by the bone marrow, but are portions of cells fragmented while circulating.

3. The cells thus fragmented are for the most part those new-formed to meet the exigencies of the situation. Such cells are in large part unable to withstand the wear and tear of function. There results a vicious circle. The anemia renders the bone marrow unable to put forth proper cells, and those it does produce are soon destroyed, thus prolonging the condition. A similar state of affairs probably exists in many human anemias.

4. The occurrence of large accumulations of microcytes and poikilocytes in the spleen of anemic and plethoric animals indicates that the organ exercises some important function in connection with these forms. The same is true of normal animals, for the findings in them are similar, though less striking.

5. The normal fate of the red corpuscles, in those species in which

<sup>7</sup> Meltzer, S. J., *Rep. Johns Hopkins Hosp. (Welch Festschrift)*, 1900, ix, 135.

phagocytosis is negligible, is to be fragmented one by one, while still circulating, to a fine, hemoglobin-containing dust. The cell fragments are rapidly removed from the blood, but their ultimate fate remains to be determined. The facts indicate that they are removed from the blood by the spleen, and under exceptional conditions, by the bone marrow.



## THE ELIMINATION OF IRON AND ITS DISTRIBUTION IN THE LIVER AND SPLEEN IN EXPERIMENTAL ANEMIA.

By HARRY DUBIN, PH.D., AND RICHARD M. PEARCE, M.D.

*(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.)*

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It has been shown in previous reports (1) from this laboratory that, in the normal dog, when the spleen is removed little or no change in the elimination of iron occurs. On the other hand, in man, splenectomy, in the course of congenital hemolytic jaundice (2) and pernicious anemia (3), is followed by a decrease in the elimination of iron equal to about 40 per cent. Moreover, in hemolytic jaundice (2) the amount of iron eliminated before splenectomy is twice as great as that taken in the food. These observations have been interpreted as indicating that in diseases of the spleen, accompanied by anemia, splenectomy removes or inhibits some factor responsible for blood destruction. This view is supported by the observation that after splenectomy there occurs also a decrease in the elimination of urobilin. On the other hand, if one compares the figures for iron elimination in anemia with those of normal individuals (4), it is found that the former fall, as a rule, within the range of the latter. This naturally brings up the question of the factors determining the storage and elimination of iron in conditions of blood destruction. Very few data concerning this subject are at hand (Table I). The only detailed observations aside from those made in this laboratory are the studies of McKelvy and Rosenbloom (5) in congenital hemolytic jaundice, and of Bayer (6), and of Roth (7) after splenectomy for Banti's disease and hemolytic anemia, respectively.

It is obviously difficult to carry out in man prolonged studies, in the different stages of anemia, of the distribution of iron in the various organs. Only end-results, as in the study of bronzed diabetes of Muir and Dunn (8), are possible. We have, therefore, in the present work attempted, by experiments on animals, to throw some light not only upon the distribution and storage of iron but also upon its elimination in various types of blood destruction due to a single or transient injury. This work we consider to be necessary as a

TABLE I.  
*Elimination of Iron in Healthy and Anemic Individuals.*

Observer.	Sex.	Age.	Iron.		Remarks.
			Intake per day.	Output per day.	
Lehmann, Mueller, Munk, Senator, and Zuntz (4).	Male.	yrs.	mg.	mg.	Professional fasters; 10 and 6 day periods respectively.
		26	Fasting.	7.3*	
		21	"	7.7	
Stockman and Greig (4).	Male.	20	6.2†	6.32‡	Healthy individuals.
			5.6	11.46	
	"	35	6.2	8.33	
	Female.	23	3.5	3.73	
Von Wendt (4).	Male (1st period).		11.0†	9.0‡	Nine periods of observation on two healthy individuals.
	(2nd " ).		6.0	11.0	
	(3rd " ).		10.0	14.0	
	(4th " ).		8.0	9.0	
	(5th " ).		17.0	42.0	
	(6th " ).		7.0	15.0	
	(7th " ).		19.0	24.0	
	(8th " ).		28.0	34.0	
	(9th " ).		27.0	32.0	
Sherman (4).	Male.		5.7§	5.5‡	Three healthy individuals.
	"		6.5	8.7	
	"		7.1	12.6	
McKelvy and Rosenbloom (5).	Female.	11	8.8†	32.51‡	Congenital hemolytic jaundice; 5 day period.
Roth (7).	Male.	26	90.0§	6.25‡	Hemolytic anemia; splenectomized 3 yrs. previously.
			150.0	4.32	
	"	37	90.0	12.18	
			200.0	33.07	
Bayer (6).	Male.	16	240.0†	19.38*	2 wks. after splenectomy for traumatic spleen rupture.
			140.0	7.41	
			130.0	14.54	
			80.0	5.92	
			300.0	26.73	

\* Feces only.

† Iron intake determined by actual analysis.

‡ Urine and feces.

§ Iron intake estimated from tables.

TABLE I—*Concluded.*

Observer.	Sex.	Age.	Iron.		Remarks.
			Intake per day.	Output per day.	
Bayer (6).	Male.	16 yrs.	mg.	mg.	
			240.0	8.40	Control; fracture of tibia.
	"	16	140.0	7.29	
			130.0	8.57	Control; osteomyelitis operation 14 days previous.
			80.0	3.57	
	Female.	19	300.0	23.49	
			130.0	13.86	Banti's disease; 2½ yrs. after splenectomy.
	"	25	130.0	10.20	Banti's disease; ½ yr. after splenectomy.
	"	27	60.0	21.46	Basedow's disease; before thymectomy.
			60.0	32.70	3 wks. after.
Goldschmidt, Pepper, and Pearce (2).	Male.	5	3.77§	8.29*	Congenital hemolytic jaundice. Before splenectomy (10 day period).
			4.56	4.11	After splenectomy (10 day period).
	Male.	40	16.5§	17.0*	Pernicious anemia. Before splenectomy (5 day period).
			16.5	10.0	2 wks. after splenectomy (4 day period).

preliminary to further experimental studies of the spleen in its relation to blood destruction, as also of the mechanism by which splenectomy in man, in the course of anemia, causes a decrease in the elimination of iron.

Our procedure, in brief, has been to determine in normal dogs and in dogs rendered anemic by the administration of various hemolytic agents (sodium oleate, toluylenediamine, and hemolytic immune serum), (a) the elimination of iron in the urine and feces, and (b) the iron content of the liver and spleen.



Observations of this nature are few in number. Corper's (9) figures for iron in the normal spleen of the dog, calculated on the basis of dry weight, are 0.26 to 0.98 per cent. Samuely (10), working likewise with the dog, gives figures for the spleen and liver, in the anemia produced by pyrodine (acetylphenylhydrazine) as 0.3921 gm. and 0.2298 gm. respectively per 100 parts of dry weight of blood- and fat-free organ and 0.4819 gm. and 0.3299 gm. per 100 parts of dry weight of fat-free but blood-containing organ. In two other animals after recovery from anemia the comparable figures were 0.1892 and 0.0721 and 0.3892 and 0.3307 gm. Tartakowky (11), after feeding iron to dogs with experimental anemia, found that the iron content of the liver rose from 0.1048 to 2.068 gm. and that of the spleen from 0.09 to 0.172 gm. Boycott and Price-Jones (12), in a study of experimental trypanosome anemia in rabbits, found the iron of the liver to be double that of normal animals, while the iron of the spleen was increased twenty times. As to the elimination of iron in normal animals, von Voit's (13) fasting dog put out 0.6 mg. per day per kilo of body weight, while Gottlieb's (14) dog on an iron-poor diet eliminated 0.34 mg. Hamburger's (15) figures with animals on a meat diet show that the output of iron in the urine is increased but slightly when large amounts of iron sulfate are fed. No other figures for the dog appear to be available.

An interesting series of experiments on rabbits, analogous to ours on the dog, has been made by Muir and Dunn (16). They produced rapid, severe anemia by the use of an hemolytic immune serum, and when the anemia was sufficiently severe, as shown by daily blood counts, the liver, spleen, kidney, stomach, and intestine were removed, after the circulating blood had been washed out, and the iron content was determined and compared with that of normal organs similarly treated. At the same time the blood volume was estimated by bleeding and a colorimetric determination of the hemoglobin content of the washings. By comparing, therefore, the iron content of normal and anemic organs, the excess in the latter was easily estimated, and by comparing hemoglobin figures during life with total blood volume at death, the amount of iron lost from the blood could be determined. Their conclusions are as follows: (1) With destruction of more than half the blood within 3 days, nearly all the iron from injured cells is deposited in the liver, spleen, and kidneys; a certain amount escapes in the urine when hemoglobinuria is present and the amount deposited in the kidneys is roughly proportional to the hemoglobinuria. (2) A third of the total iron of the blood may be deposited in the liver, spleen, and kidneys in 24 hours. Their figures show that in anemia the spleen contained three times, and the liver five times the amount of iron found in normal animals.

In a second series (17) the organs were examined when blood counts showed that the anemia had been completely repaired. The iron content of the organs was found to be only slightly above that of normal organs, indicating that the iron deposited as the result of hemolysis had been nearly all absorbed during the process of blood regeneration—presumably utilized, according to Muir and

Dunn, in the formation of new blood cells. In neither of the investigations was the elimination of iron in urine and feces determined.

### *Methods.*

As our principal object was to determine the relation between the amount of iron lost and that stored in certain organs, we limited our study to the estimation of iron in the urine and feces on the one hand, and in the liver and spleen on the other. We have used blood counts to obtain an approximate idea of the degree of blood destruction, but have not attempted to determine the actual total blood destruction. In the earlier experiments, to provide against error dependent upon a possible variation in the iron content of the organs of normal dogs, a portion of the liver and of the spleen was removed at a preliminary operation,<sup>1</sup> and the iron content determined. When later the same animal was used for the study of anemia, exact control figures for the same animal were thus at hand.

The intake of iron in food was not determined in these experiments. Previous studies (1) of iron metabolism in this laboratory have shown that with the diet used, the iron intake is relatively constant. As the diet was the same in both the control and experimental periods we have felt warranted, therefore, in ascribing changes in elimination and storage of iron as due to the experimental lesion produced.

We have also considered it unnecessary to wash out the liver and spleen in order to remove contained blood. The content of blood in each organ is relatively constant, and as it was impossible to remove the blood from the pieces of organs taken out at the preliminary control operations, we have felt that our figures would be more nearly comparable if this was not done at autopsy.

The amount of iron in the urine was determined by Wolter's (18) modification of Neumann's method, and in the feces, liver, and spleen according to Neumann's method (19), with certain modifications. A weighed amount of the substance, calculated to contain from 3 to 5 mg. of iron,<sup>2</sup> is transferred quantitatively to a 750 cc. Kjeldahl

<sup>1</sup> All operations were performed under ether anesthesia.

<sup>2</sup> For feces, weigh out 3 gm.; for spleen, 1.5 gm.; for liver, 2.5 gm.

flask; 10 gm. of potassium sulfate and 30 cc. of concentrated sulfuric acid are then added; the contents of the flask are heated over a free flame and the heating is continued for 15 minutes after the liquid assumes a straw color. After cooling<sup>3</sup> for about 5 minutes, 75 cc. of cold distilled water are slowly added, and after transfer<sup>4</sup> to a 500 cc. Erlenmeyer flask, the mixture is diluted up to about 150 cc. The rest of the analysis is carried out as described by Neumann.<sup>5</sup>

As far as accuracy is concerned, there is little to choose between this modified method of ashing and that of Neumann, for corresponding results are obtained with each. However, considering the factors of time and convenience of manipulation, a marked difference is noted in favor of our modification. With the use of sulfuric acid and potassium sulfate, the ashing is complete in from 1 to 1½ hours with practically no further attention after setting up. On the other hand, Neumann's method, involving as it does the use of nitric acid in addition to sulfuric acid, requires almost constant attention for a long period of time. Another advantage of the modification is that it does away with objectionable nitric acid fumes during ashing, and obviates the necessity of subsequent boiling to remove all traces of nitric acid.

The dogs, kept in metabolism cages, were fed on a standard diet containing 0.4 gm. of nitrogen per kilo and 70 calories per kilo of body weight. The diet consisted of beef heart, lard, bread crumbs, sugar, a little salt, and sufficient bone ash to ensure well formed feces. The food was mixed with about 300 cc. of water, the intake of the latter being constant for each day, except in such animals as received toluylenediamine *per os*. The animals were not catheterized, the urine being collected daily and preserved by cold storage; the feces were marked by carmine.

<sup>3</sup> Too much cooling should not be allowed, otherwise the addition of water will cause solidification, which is not desirable.

<sup>4</sup> In the analysis of feces, it is necessary at this point to filter off the calcium sulfate, care being taken to wash the filter thoroughly with hot water.

<sup>5</sup> Where Neumann suggests the bubbling of air through the solution to prevent overboiling, we have found it practicable to use glass beads. The water which evaporates during the boiling is replaced in order to prevent the potassium sulfate from crystallizing out during the cooling attending the filtration.

The dogs were placed on the standard diet a few days before the beginning of an experiment. After a period of 1 week, during which the normal elimination of iron in the urine and feces was determined, the animals were operated upon<sup>6</sup> and pieces of the liver and spleen were removed for the determination of the normal iron content.<sup>7</sup> Upon recovery, the animals were again placed under observation and the urine and feces analyzed to determine whether there had been any change in the iron output. Following this, an hemolytic agent was administered, and after studying the output of iron for varying periods of time (3 to 10 days), the animals were chloroformed, and the liver and spleen taken for analysis. In all cases these organs, together with the contained blood, were dried on the water bath, ground up into a fine powder, dried again at 100°C., and analyzed for iron. The feces were analyzed in a similar manner. Throughout the work all precautions were taken to prevent the introduction of extraneous iron.

Anemia was produced in one of three ways: (a) sodium oleate (Merck) given intravenously, (b) toluylenediamine (Merck) administered *per os*, and (c) hemolytic immune serum<sup>8</sup> injected intravenously.

Hemoglobin estimations and red blood cell counts were made from time to time to determine the course and severity of the anemia produced.

### *Influence of Sodium Oleate.*

Sodium oleate was selected as an hemolytic agent which produces a single injury to the red cells without continued action. The hemoglobin is set free in the circulating blood and is rapidly removed in large part by the kidney without the retention in the organs of agglutinated and injured red cells gradually undergoing disintegration.

<sup>6</sup> For the operative work we are indebted to Dr. J. E. Sweet of the Department of Surgical Research.

<sup>7</sup> As the results obtained for normal animals were so uniform, this procedure was abandoned in some of the later experiments.

<sup>8</sup> The hemolytic immune serum was prepared by injecting a rabbit repeatedly with 5 cc. of dog blood at intervals of about 5 days. 1 week after the fifth injection, the rabbit was bled and the serum collected.

The results of two of our experiments with this substance are shown in Tables II and III.

In a third experiment similar results were obtained. There was an increase of iron in the urine, corresponding to the period of hemoglobinuria, with a return to normal figures when the urine cleared. No change in the percentage of iron in the feces or liver and spleen was evident. These results indicate that in hemolysis caused by a

TABLE II.  
*Dog 1. Effect of Sodium Oleate.*

Period.	Iron* in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	mg.	per cent	per cent	per cent	
Oct. 1-7. Control period (7 days).	1.2	19.4	0.138			
Oct. 8. Control operation.				0.187	0.069	Removal of small portions of organs.
Oct. 11-17. Control period (7 days).	1.9	20.7	0.100			
Oct. 23. Sodium oleate given.						100 cc. of 1 per cent solution in vein.
Oct. 24.	8.0					Hemoglobinuria.
" 25, 26.	2.0					No hemoglobinuria.
" 26. Sodium oleate given.		14.6	0.100			200 cc. of 1 per cent solution in vein.
Oct. 27, 28.	9.5		(Oct. 23-29)			Hemoglobinuria.
" 29, 30.	2.0					No hemoglobinuria.
Nov. 1.				0.173	0.069	Animal chloroformed.

\* In this, as in all the other tables, the percentage of iron is calculated in terms of dry weight.

simple hemolytic agent the free hemoglobin is rapidly removed by the kidney and the increase of iron in the urine represents the iron of this hemoglobin. If hemoglobin does not appear in the urine the iron of the urine is not increased in amount. Analyses of the organs indicate that if hemoglobin is retained, the amount is insufficient



to change the iron content of the spleen or liver. No change is seen in the figures for the feces.

TABLE III.  
*Dog 2. Effect of Sodium Oleate.*

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	mg.	per cent	per cent	per cent	
Oct. 9-15. Control period (7 days).	1.3	20.1	0.117			
Oct. 19. Control operation.				0.175	0.112	Removal of small portions of organs.
Oct. 27. Sodium oleate given.						Hemoglobin 90 per cent; red blood cells, 5,260,000. 200 cc. of 1 per cent solu- tion in vein.
Oct. 27-29.	1.2	22.3	0.114 (Oct. 27-31)			Oct. 28. Hemoglobin 85 per cent; red blood cells 5,180,- 000. No hemoglobinuria.
Oct. 30. Sodium oleate given.	1.4					300 cc. of 1 per cent solution in vein. No hemoglobin- uria.
Oct. 31.	3.9			0.181	0.108	Hemoglobin 72 per cent; red blood cells 4,950,000. Slight hemoglobinuria. Ani- mal chloroformed.

*Influence of Toluylenediamine.*

In the second series of experiments toluylenediamine was selected because it produces a destruction of blood characterized, when moderate doses are given, by choluria rather than by hemoglobinuria, thus making it possible to determine the elimination of iron in a type of anemia in which, if iron is excreted by the kidney, the excretion is not complicated by the presence of free hemoglobin. Moreover, toluylenediamine, unlike sodium oleate, which produces its



effect in a short space of time, has a more prolonged cumulative effect and destroys red cells, according to Joannovics and Pick (20) and Maidorn (21), in part at least, through an hemolytic agent which is intimately connected with the products of fatty degeneration. The mechanism of blood destruction is, therefore, of a more complicated type than that due to sodium oleate. An experiment of this type, with intoxication for 1 week only, is shown in Table IV.

TABLE IV.  
*Dog 3. Effect of Toluylenediamine.*

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	mg.	per cent	per cent	per cent	
Nov. 25-Dec. 1. Control period (7 days).	1.2	19.7	0.101			
Dec. 8. Control operation.				0.187	0.104	Removal of small portions of organs.
Dec. 11-17. Toluyl- enediamine for 7 days.	1.6	21.6	0.108			0.2 gm. of toluylenediamine <i>per os</i> daily. Choloria daily; no hemoglobinuria.
Dec. 18.				0.283	0.146	Animal chloroformed.

In this animal, despite the fact that a moderately severe anemia was present, as shown by 3,300,000 red cells and 58 per cent of hemoglobin on December 17, practically no change in the elimination of iron occurred. On the other hand, the figures show a considerable increase in the percentage of iron in the liver and spleen.

In a second experiment in every way similar, except that toluylenediamine was administered for 9 days instead of 7, analogous results were obtained. The iron in the urine in the period of anemia was 1.6 mg. daily as compared with 1.4 mg. for the control period, and the percentage of iron in the feces, 0.110 and 0.103 for the respective periods. The iron in the spleen and liver, respectively, in the anemia period amounted to 0.299 and 0.154 per cent, as compared with 0.190 and 0.104, respectively, for the normal. The urine

of this animal was constantly bile-stained and the hemoglobin content of the blood fell from 86 to 58 per cent.

In another pair of experiments, toluylenediamine was given for 1 week and discontinued for the same period. The object of these experiments was to determine whether a delayed elimination of iron occurred simultaneously with a resorption of iron from the liver and spleen.

In these and some of the later experiments the preliminary control operation, for the purpose of obtaining tissue for iron analysis, was not done. As may be seen in Table V, the figures for iron in tissues obtained by this preliminary procedure are fairly uniform, averaging 0.104 per cent for the liver and 0.185 for the spleen. In view of this uniformity, the control operation was, therefore, not always done as a necessary part of each experiment, and it was assumed that the normal iron content of the liver is never more than 0.112 per cent, and that of the spleen never more than 0.190 per cent. Reference to Table V will show that these figures are secured by excluding only one extremely low figure, that for the liver of Dog 6.

TABLE V.

*Per Cent of Iron in the Liver and Spleen of the Normal Dog.*

Dog No.	Liver.	Spleen.
4	0.104	0.187
5	0.104	0.190
6	0.069 (?)	0.187
7	0.098	0.180
8	0.108	0.187
9	0.100	0.188
10	0.112	0.175
Average.....	0.104	0.185

The results of an experiment in which a recovery period of 1 week was allowed after the administration of toluylenediamine are shown in Table VI.

In a second experiment carried out on exactly the same lines, the urine figures in mg. for the three periods were 1.4, 1.6, and 1.5; those for the feces were 18.6, 20.7, and 19.3 with percentages of 0.107, 0.110, and 0.100. The percentage of iron in the liver was 0.132 and in the spleen 0.233. The urine was bile-stained throughout the

period of intoxication. The blood examination gave 101 per cent of hemoglobin in the first period, 70 at the end of the second, and 98 at the end of the third.

These experiments show no evidence of immediate or delayed iron elimination by the kidney or intestine and the figures for iron in the liver and spleen, definitely higher than those for normal organs, would appear to indicate that the increased amount retained, as the result of blood destruction, is not readily given up by these organs.

TABLE VI.

*Dog 11. Toluylenediamine with an Interval for Recovery.*

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	mg.	per cent	per cent	per cent	
Jan. 19-25. Control period (7 days).	1.5	16.6	0.106			Jan. 26. Hemoglobin 102 per cent; red blood cells 5,240,000.
Jan. 26-Feb. 1. Toluylenediamine for 7 days.	1.4	18.2	0.100			0.2 gm. of toluylenediamine daily. Urine constantly bile-stained. Feb. 1. Hemoglobin 70 per cent; red blood cells 3,580,000.
Feb. 2-8. Recovery period.	1.7	17.2	0.103			
Feb. 9.				0.253	0.140	Hemoglobin 95 per cent; red blood cells 4,990,000. Animal chloroformed.

### *Influence of Hemolytic Immune Serum.*

Hemolytic serum was used in part on account of its definite hemolytic effect, but chiefly because of its agglutinative effect on red cells and their consequent accumulation in the liver, spleen, and lymph nodes. The red cells responsible for the sporogenous tumor of the spleen and enclosed in the endothelial cells of the liver and lymph nodes represent an accumulation of hemoglobin gradually disintegrating and therefore altering profoundly the iron content of these organs. All injections of serum<sup>8</sup> were made intravenously.

Two experiments (Table VII) support the histological evidence that agglutinated and altered red cells are held in the organs immediately after the administration of an agglutinative and hemolytic

TABLE VII.  
*Effect of Hemolytic Serum.*

Dog No.	Period.	Iron in.		Remarks.
		Liver.	Spleen.	
		<i>per cent</i>	<i>per cent</i>	
12	Nov. 3. Control operation.	0.098	0.180	0.5 cc. of serum per kilo. Died in 3 hrs.
	" 9. Serum given.	0.177	0.287	
13	Nov. 9. Control operation.	0.100	0.188	0.4 cc. of serum per kilo. Died in 3 hrs.
	" 11. Serum given.	0.176	0.301	

TABLE VIII.  
*Dog 14. Effect of Hemolytic Serum.*

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Liver.	Spleen.	
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Dec. 6-12. Control period (7 days).	1.7	11.0	0.110			
Dec. 15. Control operation.				0.108	0.187	Hemoglobin 105 per cent; red blood cells 9,220,000.
Dec. 17-23. Period of anemia* with 4 days of hemoglobinuria.	6.1	31.2	0.120			Dec. 22. Hemoglobin 55 per cent; red blood cells 5,630,000.
Dec. 24.				0.204	0.447	Animal chloroformed.

\* On Dec. 17, the animal received 0.06 cc. of serum per kilo and on Dec. 18, 0.09 cc. per kilo. These injections were followed by the appearance of bile pigment in the urine. On Dec. 19, 0.18 cc. of serum per kilo was administered; this was followed by hemoglobinuria persisting for 4 days.

serum. The dogs in question died within 3 hours after receiving the serum, and the increase in iron content cannot therefore be ex-

plained by a deposition of free iron but as due to the masses of erythrocytes held in the endothelial cells and small vessels of the organs. It should be noted that in these two experiments we returned to the practice of performing a preliminary control operation in order to determine the exact amount of iron in the normal organs. The estimation of iron in the urine and feces was impossible on account of the short time the animals survived the injection.

TABLE IX.  
*Dog 15. Effect of Hemolytic Serum.*

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Liver.	Spleen.	
	mg.	mg.	per cent	per cent	per cent	
Control period (7 days).	1.8	21.1	0.098			Mar. 28. Hemoglobin 98 per cent; red blood cells 5,120,000.
Mar. 29-Apr. 4. Period of anemia* with 3 days of hemoglobinuria.	11.1	30.8	0.105			Mar. 30. Hemoglobin 50 per cent; red blood cells 3,980,000.
Apr. 5-11. Choluria but no hemoglobinuria.	1.5	23.6	0.103			Apr. 10. Hemoglobin 40 per cent.
Apr. 17.				0.245	0.490	Hemoglobin 30 per cent; red blood cells 2,780,000. Animal chloroformed.

\*On Mar. 29, the injection of 0.17 cc. of serum per kilo caused the appearance of bile pigment in the urine; 0.23 cc. on Mar. 31 caused hemoglobinuria lasting 3 days, and the persistence of bile pigment until the end of the experiment.

In two other experiments of the same type, with smaller doses of serum (0.25 cc. per kilo) and without preliminary operation, the animals were killed by chloroform at the end of 18 and 24 hours respectively. The same increase of iron in the liver and spleen was found—0.176 and 0.178 per cent for the respective livers and 0.318 and 0.307 per cent for the spleens.

In a fifth experiment (Table VIII), with preliminary operation,

the analysis of the feces shows that although the total iron in the feces is increased, the percentage remains practically unchanged. The increase of iron in the urine is due to the presence of hemoglobin. As usual, the iron content of the liver and spleen is increased.

In the final experiment (Table IX) of the series, the observations were continued for 2 weeks through a period of prolonged severe anemia. This experiment shows clearly that the iron of the urine is increased only in the presence of hemoglobinuria. During the period of April 5 to 11, blood disintegration continued, as shown by the continued fall of hemoglobin and red cell count and by the presence of bile in the urine, but the iron content of the urine was lower than in the control period. The total elimination of iron in the feces was appreciably higher in the first period of anemia, but the percentage was altered but little. These variations are to be explained, as also those given in Table VIII, by differences in the bulk of feces. The figures for iron in the liver and spleen showed the usual increase.

#### SUMMARY.

Blood destruction due to a single injury, as by sodium oleate, or acting through a short period of time, as by toluylenediamine or hemolytic immune serum, is not characterized, in the absence of hemoglobinuria, by an increased elimination of iron in the urine. This holds, not only for the evanescent injury caused by sodium oleate, but also for the severe type caused by hemolytic immune serum, in which a progressive destruction of the blood may persist for 2 weeks or more with constant evidence of the disintegration of erythrocytes as shown by bile pigment in the urine. This finding is in accord with previous investigations of anemia in both man and animals.

Likewise, no striking increase is evident, under such circumstances, in the percentage of iron excreted in the feces. The total amount of iron in the feces has been notably increased in two experiments with hemolytic serum, but as the percentage was not appreciably altered, the difference depends presumably on variations in the bulk of feces rather than upon increased elimination.

This evidence of the power of the body to conserve the iron re-



sulting from erythrocytic disintegration is further emphasized by the increased storage of iron in the liver and spleen. This storage is not evident in the slight transient injury caused by sodium oleate, but in the more prolonged anemia due to toluylenediamine and hemolytic serum, the iron content of the spleen and liver is greatly increased, and in the case of anemia due to hemolytic serum is more than doubled.

In view of this definite evidence of the power of the animal body to conserve iron, it is obvious that in the hemolytic anemias in man characterized by excessive elimination of iron in the feces, some other factor than mere blood destruction is operative. Theoretically, it may be assumed that it is a disturbance of the mechanism concerned in the retention or conservation of iron. This phase of the problem we are now studying in experimental types of chronic progressive anemia due to a persisting and ever active cause; and we trust that the studies may throw some light on the iron exchange in anemia and in particular upon the class of hemolytic anemias so markedly benefited by splenectomy.

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# A STUDY OF THE ACIDOSIS, BLOOD UREA, AND PLASMA CHLORIDES IN URANIUM NEPHRITIS IN THE DOG, AND OF THE PROTECTIVE ACTION OF SODIUM BICARBONATE.

By KINGO GOTO, M.D.

*(From the William Pepper Laboratory of Clinical Medicine of the University of Pennsylvania, Philadelphia.)*

PLATES 57 AND 58.

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## HISTORICAL.

Numerous investigations have been made concerning the relation between nephritis and the alkalinity of the blood. The earliest studies were made by titration of the blood serum or of a dialysate of the serum with a weak acid, usually phosphoric or tartaric acid with litmus paper as an indicator. By such a method von Jaksch (1) noted a decrease in the alkalinity of the blood in uremia and concluded that the diminished alkalinity of the blood is a factor in inducing some of the phenomena of uremic intoxication. Brandenburg (2, 3), using a similar method, confirmed von Jaksch's finding. The methods employed by these investigators are, however, admittedly inadequate. In 1912 Straub and Schlayer (4), using Haldane's method, found a diminution in the carbon dioxide content of the alveolar air in uremia and thus established on a secure basis the association of acidosis with uremia. Von Noorden (5), however, considered that although a slight grade of acidosis may be present in uremia, it represents merely an associated phenomenon (perhaps the result of inanition) and not the underlying cause of the intoxication. Sellards (6) observed that a larger quantity of sodium bicarbonate must be ingested by certain nephritics, in order to render their urine alkaline, than is the case with normal individuals. This phenomenon he called "increased tolerance to sodium bicarbonate" and concluded that it indicates a condition of acidosis. In 1913 Palmer and Henderson (7), applying the principle of Sellards, but with a more highly developed method for determining the acidity of the urine, found evidence of some degree of acidosis in many pathological conditions. Among these were certain acute infections, severe anemias, the cachexias of malignant neoplasms, and certain types of nephritis.

There are also numerous investigations concerning the effect of alkali and acid upon the alkalinity of the blood and of the urine. Miquel (8) noted a diminution of the alkalinity of the blood of normal animals following the administration

of acids. Gaethgens (9), on the other hand, observed a rapid excretion of the acid introduced without detecting any depletion of the bases of the blood. Lassar (10) using the tartaric acid titration method with litmus paper as an indicator reported a slight, but unquestionable decrease in the alkalinity of the blood of a dog to which acid had been administered. A more reliable method was that employed by Walter (11) in 1877, who made use of the fact that the carbon dioxide content of the blood is proportional to the content of available base in the blood. He demonstrated that when an animal is treated by injecting an acid into the blood stream, the blood undergoes a diminution in its content of base as shown by its diminished carbon dioxide content. He also showed that under such conditions there is an increased ammonia excretion in the urine and eventually a respiratory death, which may be delayed by the administration of soda. Von Hösslin (12) found an intimate relation between the acidity of the urine as determined by the method of Moritz and the amount of albumin and casts in the urine. He observed that following a lowered acidity of the urine after administration of soda, the albumin in the urine as well as casts diminishes, and, furthermore, that such lowered acidity is accompanied by an improvement in the renal functional capacity as indicated by a better excretion of sodium chloride. Von Hösslin (13), therefore, advocated the administration of sodium bicarbonate in order to reduce the acidity of the urine in nephritis. He pointed out that the initial acidity of the urine is no gauge of the amount of soda that must be given to render the urine alkaline. Henderson and Palmer (14) have investigated the effect of acid ingestion upon the hydrogen ion concentration in urine and found a constant increase of acidity in the urine after the ingestion of considerable amounts of acid, but they were not able to produce a urine of an acidity as great as that common in many pathological conditions. Scheltema (15) also obtained favorable effects from the administration of alkali to nephritic individuals. Henderson and Palmer (16), in a study of the factors of acid excretion in nephritis, found "first, that the urinary concentration of ionized hydrogen is, in a statistical sense, increased in the various forms of nephritis; and secondly that such pathological states are frequently marked by a condition of acidosis." They found (17) a renal retention of alkali in those cases of nephritis in which excretion of ammonia is diminished in the urine.

#### EXPERIMENTAL.

The present investigation was undertaken to study the development of acidosis in nephritis produced by uranium nitrate and the relation of this acidosis to the changes in urea and chlorides of the blood, and also to study the effect of administration of sodium bicarbonate upon all these factors. In these experiments the following determinations were made: (1) the carbon dioxide content of



the plasma and the hydrogen ion concentration of the serum, (2) the urea nitrogen of the blood, (3) the chlorides of the plasma, and (4) the reaction of the urine and its content of albumin and casts. For the carbon dioxide content of the plasma, the Van Slyke-Stillman-Cullen (18) method was used; for the hydrogen ion concentration of the serum, Marriott's modification of the Levy-Rowntree-Marriott method (19, 20); for urea determinations, the Van Slyke-Cullen method (21, 22); and for the plasma chlorides, the method of McLean and Van Slyke (23). In every instance duplicate determinations were done.

The blood for these determinations, except for the hydrogen ion concentration of the serum, was obtained by drawing the blood from the external jugular vein through a tube passing to the bottom of a centrifuge tube containing either sodium oxalate or potassium oxalate crystals and a layer of paraffin oil which, floating on the surface of the blood, excluded contact with the air. The amount of oxalate employed was about 1 per cent by weight of the amount of blood. A portion of the whole blood was removed for urea determination and the remainder was centrifuged. Of the plasma so obtained, 1 cc. was transferred directly from beneath the paraffin oil to the Van Slyke burette for determination of its carbon dioxide content. The results of these determinations are given in the tables in the columns headed "direct." The remainder of the plasma was removed from the cells and used for chloride determination and for the carbon dioxide content after saturation in an atmosphere of 5.5 per cent carbon dioxide at room temperature. This saturation was performed by introducing about 3 cc. of the plasma into a 250 cc. separatory funnel and filling this with normal alveolar air by exhaling deeply five times through the funnel. The funnel was then closed and the plasma shaken in this atmosphere for 1 minute. 1 cc. portions were then transferred to the burette for analysis. The results of these determinations are given in the columns headed "funnel." These readings were always completed within an hour of the drawing of the blood. For the hydrogen ion concentration of the serum, blood was drawn directly from the vein into a small test-tube and the serum separated by centrifuging. In addition, plasma was examined by Marriott's method. The columns headed



"direct" represent the reading obtained at the close of dialysis; the columns headed "A. B." are the readings after the removal of carbon dioxide from the dialysate by aeration.

*Plasma Carbon Dioxide Content, Blood Urea, and Plasma Chlorides in Dogs with Uranium Nephritis.*

Since it has been noted by MacNider (24) that the age of a dog has an important bearing on the degree of nephritis caused by uranium nitrate, dogs of the same age have been used as far as possible. The importance of diet in determining the toxicity of uranium nitrate has been shown by Opie (25). Animals have been shown to be more susceptible to this poison when kept upon a diet rich in meat than when upon a diet rich in carbohydrates. For this reason, the animals employed have received a constant diet of milk and dog biscuit with no meat. The animals were fed daily at 5 p.m. In regard to the method of injection, the crystalline uranium nitrate has been dissolved in distilled water and given to the dogs, at the first injection in the proportion of 0.015 gm. per 10 kilos of body weight. After an interval of 20 days another injection of the same dose was given, followed by two subsequent injections at 10 day intervals. The amount of uranium given was increased in the third and fourth injections; in the third injection 0.0185 gm. and in the fourth injection 0.045 gm. per 10 kilos of body weight. The object of the increased doses was to produce severe nephritis, in which the carbon dioxide content, urea, and chlorides of the blood might undergo a more marked alteration. Finally, at the end of the experiment, the animals were killed and the kidneys examined. Four dogs were used in the experiments. They were bled at 9 a.m. on the days when the blood was studied. Three preliminary examinations were made on each dog before administering either uranium or soda. The results of these examinations are shown in Table I. The results following the administration of uranium to Dogs 1 and 2 are shown in Tables II and III and Text-figs. 1, 2, and 3.

In Dog 1, after the first injection of uranium, the carbon dioxide content of the plasma underwent a considerable diminution in the course of a week, and, at the same time, the urea and chlorides of

TABLE I.  
*Normal Dogs.*

	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
	Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin	Casts.
			Di-rect.	A. B.	Di-rect.	A. B.					
	<i>per cent</i>	<i>per cent</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>mg.</i>	<i>mg.</i>			
Dog 1	55	58	7.5	8.0	7.4	7.9	11	5.6	Acid.	—	—
	63	66	7.9	8.1	7.5	7.8	13	5.9	“	—	—
	62	65	7.8	8.0	7.4	7.9	12	6.0	“	—	—
Average.....	60	63	7.7	8.0	7.4	7.9	12	5.8			
Dog 2	56	61	7.4	7.9	7.3	7.7	14	5.4	Acid.	—	—
	60	65	7.8	8.0	7.4	7.7	13	5.9	“	—	—
	61	65	7.6	8.0	7.4	7.8	12	5.6	“	—	—
Average.....	59	64	7.6	8.0	7.4	7.7	13	5.6			
Dog 3	55	62	7.8	7.9	7.6	7.9	13	5.8	Acid.	—	—
	56	62	7.9	8.0	7.6	8.0	13	5.9	“	—	—
	57	64	7.9	8.0	7.4	8.0	13	5.8	“	—	—
Average.....	56	63	7.9	8.0	7.6	8.0	13	5.8			
Dog 4	55	60	7.8	7.9	7.3	7.9	13	5.5	Acid.	—	—
	58	65	7.8	7.9	7.4	7.7	15	6.0	“	—	—
	60	66	7.9	8.0	7.4	7.8	14	5.8	“	—	—
Average.....	58	64	7.8	7.9	7.4	7.8	14	5.8			

*Extent of Variability.*

Van Slyke method.		Marriott method.				Urea nitrogen.	Chlorides.
Direct.	Funnel.	Serum.		Plasma.			
		Direct.	A. B.	Direct.	A. B.		
<i>per cent</i>	<i>per cent</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>mg.</i>	<i>mg.</i>
55-63	58-66	7.4-7.9	7.9-8.1	7.3-7.6	7.7-8.0	11-15	5.4-6.0

TABLE II.

Dog 1. Weight 8 kilos.

First injection, 2 p.m., July 3, 1916.

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.			
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.	
				Direct.	A. B.	Direct.	A. B.						
		per cent	per cent	log.	log.	log.	log.	mg.	mg.				
Average . . . . .		60	63	7.7	8.0	7.4	7.9	12	5.8	Acid.	—	—	
1	1916 July 3	12 mg. of uranium nitrate at 2 p.m.											
2	July 4												
3	" 5	57	63	7.8	7.9	7.1	7.6	21	6.3	Acid.	Tr.	—	
4	" 6									"	"	—	
5	" 7	53	56	7.7	7.8	7.3	7.7	28	6.7	"	"	+	
6	" 8	48	55	7.1	7.8	7.0	7.8	32	6.7	"	++	+	
7	" 9									"	+	+	
8	" 10	45	50	7.1	7.7	6.9	7.9	36	6.8	"	+	+	
9	" 11	40	46	7.0	7.8	6.9	7.8	23	7.0	"	+	+	
10	" 12									"	+	+	
11	" 13	55	60	7.2	7.9	7.1	7.8	21	6.0	"	+	—	
12	" 14									"	+	—	
13	" 15	60	65	7.5	7.7	7.3	7.6	19	6.3	"	+	—	
14	" 16									"	+	—	
15	" 17									"	Tr.	—	
16	" 18	58	60	7.7	8.0	7.3	7.8	21	5.8	"	"	—	
17	" 19									"	"	—	
18	" 20	60	65	7.8	7.9	7.4	7.7	16	5.8	"	"	—	
19	" 21									"	"	—	
20	" 22	63	65	7.9	8.0	7.4	7.9	16	6.2	"	"	—	
21	" 23												
22	July 24	12 mg. of uranium nitrate at 9 a.m.									Acid.	Tr.	—
23	July 25									Acid.	Tr.	—	
24	" 26			7.7	7.8	7.3	7.4	23	6.6	"	+	—	
25	" 27									"	+	+	
26	" 28			7.6	7.8	7.3	7.8	26	6.6	"	++	+	
27	" 29									"	++	+	
28	" 30									"	++	+	
29	" 31			7.5	7.8	7.3	7.8	20	6.3	"	++	+	
30	Aug. 1									"	+	+	
31	" 2			7.6	7.8	7.4	7.7	21	6.2	"	+	—	
32	" 3									"	Tr.	—	
33	" 4	60	65	7.7	7.8	7.4	7.6	21	6.1	"	"	—	

TABLE II—*Concluded.*

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.
				Direct.	A. B.	Direct.	A. B.					
33	Aug. 4	15 mg. of uranium nitrate at 9 a.m., after bleeding.								Acid.	Tr.	—
34	Aug. 5	<i>per cent</i>	<i>per cent</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>mg.</i>	<i>mg.</i>	Acid.	Tr.	—
35	" 6									"	"	—
36	" 7									"	+	—
37	" 8	48	52	7.8	7.8	7.4	7.8	21	6.5	"	+	—
38	" 9									"	+	—
39	" 10	46	50	7.6	7.8	7.2	7.6	22	6.9	"	+	—
40	" 11									"	+	+
41	" 12	49	55	7.9	8.0	7.4	7.8	22	6.7	"	++	+
42	" 13									"	+	—
43	" 14									"	+	—
44	Aug. 15	36 mg. of uranium nitrate at 9 a.m.								Acid.	+	—
45	Aug. 16	49	53	7.6	7.9	7.4	7.8	29	7.0	Acid.	+	+
46	" 17	45	47	7.4	7.8	7.2	7.7	29	7.1	"	++	+
47	" 18	42	46	7.2	7.8	7.1	7.7	26	7.3	"	++	+
48	" 19	40	47	7.2	7.8	7.1	7.6	25	7.3	"	++	+
49	" 20	45	49	7.3	7.8	7.1	7.6	26	7.4	"	++	+
50	" 21									"	+	+
51	" 22	52	56	7.7	7.8	7.2	7.6	25	7.1	"	+	+

the blood exhibited an increase associated with the appearance of albumin and casts in the urine. It will be noted that the minimum of the plasma carbon dioxide content coincides approximately with the maximum of the blood urea and plasma chlorides in time of occurrence. At about this period casts also appeared in the urine. During the 3rd week after the first injection, the plasma carbon dioxide content returned to its normal level, and on the 20th day, the values for plasma chlorides and blood urea nitrogen were 6.2 and 16 respectively. The chlorides, however, had three times been within normal limits between the 11th and 20th days. Urine at

TABLE III.

Dog 2. Weight 27 kilos.

First injection, 2 p.m., July 3, 1916.

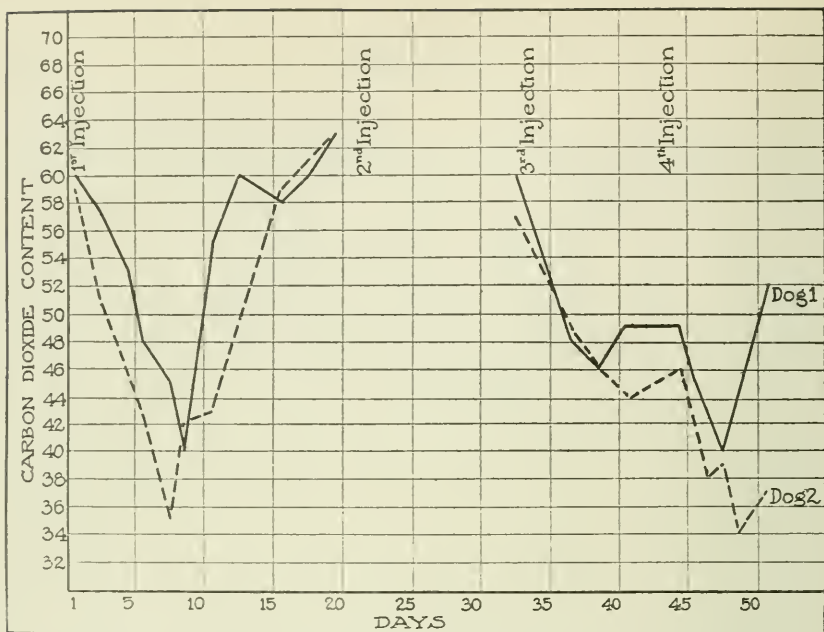
Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.			
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.	
				Direct.	A. B.	Direct.	A. B.						
		per cent	per cent	log.	log.	log.	log.	mg.	mg.				
Average.....		59	64	7.6	8.0	7.4	7.7	13	5.6	Acid.	—	—	
1	<sup>1916</sup> July 3	40 mg. of uranium nitrate at 2 p.m.											
2	July 4									Acid.			
3	" 5	51	58	7.8	8.0	7.3	7.8	21	6.4	"	Tr.	—	
4	" 6									"	"	—	
5	" 7	46	48	7.6	7.8	7.4	7.6	23	6.6	"	+	+	
6	" 8	43	47	6.9	7.7	6.8	7.6	25	7.2	"	++	+	
7	" 9									"	++	+	
8	" 10	35	39	6.9	7.4	6.8	7.3	42	7.3	"	++	+	
9	" 11	42	45	7.1	7.8	7.0	7.5	41	6.9	"	++	+	
10	" 12									"	++	+	
11	" 13	43	46	7.1	7.8	7.0	7.5	42	6.9	"	+	+	
12	" 14									"	+	+	
13	" 15	49	52	7.5	7.7	7.3	7.5	41	6.6	"	+	+	
14	" 16									"	+	—	
15	" 17									"	Tr.	—	
16	" 18	59	62	7.7	7.9	7.4	7.9	36	6.1	"	"	—	
17	" 19									"	"	—	
18	" 20	61	63	7.9	8.1	7.3	7.6	26	6.0	"	"	—	
19	" 21									"	"	—	
20	" 22	63	65	7.8	8.0	7.4	7.8	16	5.9	"	"	—	
21	" 23												
22	July 24	40 mg. of uranium nitrate at 9 a.m.									Acid.	Tr.	—
23	July 25									Acid.	Tr.	—	
24	" 26			7.8	7.9	7.4	7.8	22	5.9	"	+	—	
25	" 27									"	+	+	
26	" 28			7.6	7.8	7.3	7.7	26	6.3	"	++	+	
27	" 29									"	++	+	
28	" 30									"	++	+	
29	" 31			7.4	7.7	7.3	7.7	26	6.5	"	++	+	
30	Aug. 1									"	+	+	
31	" 2			7.5	7.7	7.3	7.6	20	6.1	"	+	?	
32	" 3									"	+	?	
33	" 4	57	60	7.6	7.7	7.3	7.5	20	6.1	"	+	+	

TABLE III—*Concluded.*

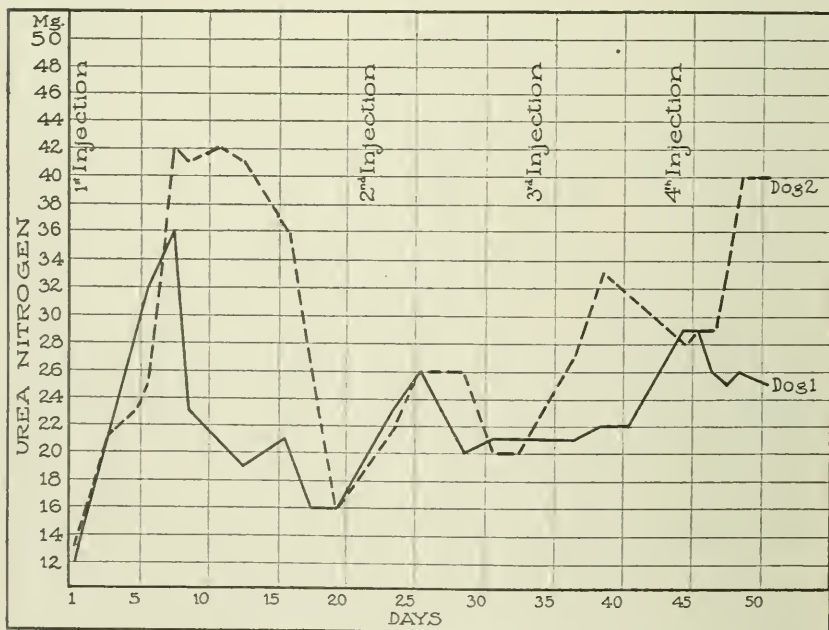
Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.			
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.	
				Direct.	A. B.	Direct.	A. B.						
33	Aug. 4	50 gm. of uranium nitrate at 9 a.m., after bleeding.											
34	Aug. 5	<i>per cent</i>	<i>per cent</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>mg.</i>	<i>mg.</i>	Acid.	+	+	
35	" 6									"	+	+	
36	" 7									"	+	+	
37	" 8	49	53	7.7	7.7	7.4	7.7	27	6.7	"	++	+	
38	" 9									"	++	+	
39	" 10	46	48	7.6	7.8	7.3	7.7	33	7.0	"	++	+	
40	" 11									"	++	?	
41	" 12	44	46	7.7	7.8	7.3	7.8	31	6.9	"	+	+	
42	" 13									"	+	+	
43	" 14									"	+	+	
44	Aug. 15	120 mg. of uranium nitrate at 9 a.m.									Acid.	+	+
45	Aug. 16	46	49	7.6	7.7	7.3	7.6	28	7.0	Acid.	+	+	
46	" 17	42	45	7.3	7.7	7.2	7.6	29	7.3	"	+	+	
47	" 18	38	40	7.1	7.7	7.0	7.5	29	7.2	"	++	+	
48	" 19	39	45	7.1	7.6	7.0	7.5	34	7.3	"	++	+	
49	" 20	34	40	7.1	7.7	7.0	7.6	40	7.3	"	++	+	
50	" 21									"	++	+	
51	" 22	37	43	7.1	7.6	7.1	7.5	40	7.1	"	++	+	

this time still showed the presence of a trace of albumin, although casts were absent. After the second injection of uranium similar changes occurred in the blood urea and plasma chlorides, but less pronounced in degree; the carbon dioxide content could not be studied at this time as the only burette at hand was broken. After the third and larger injection, a marked reduction of the carbon dioxide content occurred and a synchronous marked rise of plasma chlorides; no significant change was noted in the blood urea. It may be noted that casts in the urine appeared rather late. After the fourth injection, which was given before the blood had returned to a normal

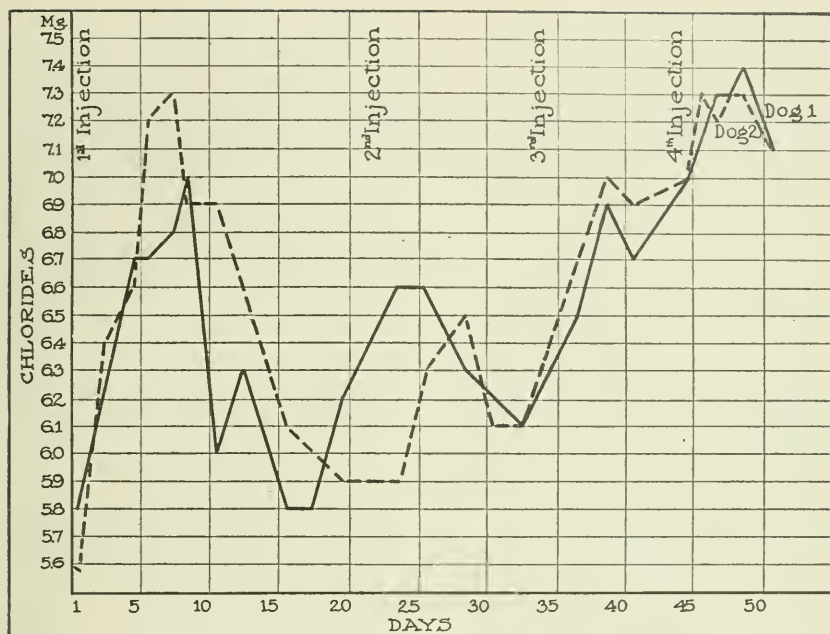




TEXT-FIG. 1. Curves showing the carbon dioxide content of the plasma of Dogs 1 and 2 after injections of uranium nitrate. No soda administered.



TEXT-FIG. 2. Curves showing the urea nitrogen in 100 cc. of blood in Dogs 1 and 2 after injections of uranium nitrate. No soda administered.



TEXT-FIG. 3. Curves showing the chlorides in 1 cc. of the plasma in Dogs 1 and 2 after injections of uranium nitrate. No soda administered.

condition, the minimum of plasma carbon dioxide content and the maximum of plasma chloride coincided approximately in their occurrence about the 4th day after injection. During this last period the chlorides in the blood were much higher and the albumin in the urine was much greater in quantity than after the previous injections. The increase in the quantity of blood urea, however, was less than after the first injection.

In the second dog nearly identical relations in the carbon dioxide content, urea, and chlorides of the blood and albumin and casts in the urine were observed as in the first dog. We may, however, note the following slight differences. After their reappearance 3 days after the second injection, the urinary casts persisted throughout the experiment. After each injection the carbon dioxide content, the chloride content, and the blood urea showed greater disturbances than in the first dog. This is prob-

ably to be attributed to the much larger absolute dose of uranium, although the same dose was used per kilo. We may conclude from these experiments (Dogs 1 and 2) that the plasma carbon dioxide content undergoes a considerable diminution in uranium nephritis, while the plasma chlorides and blood urea increase, and, furthermore, that the occurrence of the minimum content of carbon dioxide coincides approximately with the occurrence of the maximum content of chlorides and of urea in the blood. Both the carbon dioxide content and plasma chlorides returned nearly to their normal condition 2 to 3 weeks after the first injection. Urea, on the other hand, did not return quite to its normal level at any period, although 3 weeks after the first injection, the change from the normal value was slight. Albumin after appearing did not disappear entirely from the urine, although casts occasionally did.

*Influence of Sodium Bicarbonate on the Plasma Carbon Dioxide Content, Blood Urea, and Plasma Chlorides in Dogs with Uranium Nephritis.*

For this experiment Dogs 3 and 4 were used. Each dog received through a stomach tube 1 gm. of sodium bicarbonate dissolved in 10 cc. of water per kilo of body weight at 9 a.m. throughout the entire period of the experiment. On the 3rd day uranium nitrate was injected. The amounts of the uranium per kilo and the intervals between succeeding injections were exactly the same as in the experiment on Dogs 1 and 2. The blood was taken at 9 a.m., before administering the soda. The results are shown in Tables IV and V and Text-figs. 4, 5, and 6. While the dogs were receiving sodium bicarbonate alone, that is, before the giving of uranium, a considerable increase of the plasma carbon dioxide content was observed. Following the first uranium injection this carbon dioxide content showed a decrease and at the same time there occurred an increase in the plasma chlorides and blood urea, as in Dogs 1 and 2.

The relative decrease in the carbon dioxide content following uranium in the dogs receiving soda is comparable with that in the dogs receiving no soda, but the absolute level reached was not so low, because of the higher level already existing when the uranium

TABLE IV.

Dog 3. Weight 13.5 kilos.

First injection, 9 a.m., July 5, 1916

July 3. 12 a.m. 13.5 gm. of sodium bicarbonate + 135 cc. of water.

From July 4 sodium bicarbonate + water was given every day, after bleeding.

Day.	Date.	Van Slyke method.		Marriott method				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.
				Direct.	A. B.	Direct.	A. B.					
		<i>per cent</i>	<i>per cent</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>mg.</i>	<i>mg.</i>			
Average . . . . .		56	63	7.9	8.0	7.6	8.0	13	5.8	Acid.	—	—
1	1916 July 3, 2 p.m.	69	71	7.8	8.0	7.5	7.9	13	5.6	Alkaline.	—	—
2	“ 4											
3	July 5	20 mg. of uranium nitrate at 9 a.m.								Alkaline.	—	—
4	July 6	66	68	7.8	8.0	7.4	7.9	14	6.1	Alkaline.	Tr.	—
5	“ 7	56	60	7.7	8.0	7.3	7.9	15	6.2	“	“	—
6	“ 8	55	60	7.4	7.9	6.9	7.8	19	6.2	“	“	—
7	“ 9									“	“	—
8	“ 10	47	52	7.3	7.7	6.9	7.8	26	6.8	“	“	+
9	“ 11									“	“	+
10	“ 12	48	50	7.2	7.8	6.9	7.6	29	6.7	“	+	+
11	“ 13									“	+	+
12	“ 14	55	58	7.4	7.8	7.0	7.6	30	6.0	“	+	+
13	“ 15									“	+	—
14	“ 16									“	+	—
15	“ 17	61	64	7.7	7.9	7.4	7.9	29	6.4	“	Tr.	—
16	“ 18									“	“	—
17	“ 19	65	68	7.9	8.1	7.4	7.9	32	6.0	“	“	—
18	“ 20									“	“	—
19	“ 21	70	75	8.0	8.1	7.5	7.9	16	6.0	“	“	—
20	“ 22									“	“	—
21	“ 23									“	“	—
22	“ 24	70	73	7.9	8.1	7.5	7.9	22	5.6	“	“	—
23	“ 25											
24	“ 26											
25	“ 27			8.0	8.1	7.4	7.8	25	5.9	Alkaline.	Tr.	—

TABLE IV—*Concluded.*

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.			
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.	
				Direct.	A. B.	Direct.	A. B.						
25	July 27	20 mg. of uranium nitrate at 9 a.m., after bleeding.											
26	July 28	<i>per cent</i>	<i>per cent</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>mg.</i>	<i>mg.</i>	Alkaline.	+	—	
27	" 29			7.8	8.0	7.4	7.9	26	6.5	"	+	—	
28	" 30									"	+	—	
29	" 31									"	+	—	
30	Aug. 1			7.5	7.8	7.3	7.5	17	6.0	"	+	—	
31	" 2									"	+	—	
32	" 3			7.6	7.8	7.2	7.5	15	5.8	"	+	—	
33	" 4									"	+	—	
34	" 5	62	55	7.6	7.8	7.3	7.6	15	6.0	"	Tr.	—	
35	" 6									"	"	—	
36	Aug. 7	25 mg. of uranium nitrate at 9 a.m.									Alkaline.	Tr.	—
37	Aug. 8									Alkaline.	Tr.	—	
38	" 9									"	+	—	
39	" 10	52	55	7.5	7.8	7.3	7.6	28	6.3	"	+	—	
40	" 11									"	+	—	
41	" 12	49	52	7.3	7.8	7.2	7.6	23	6.2	"	+	—	
42	" 13									"	+	—	
43	" 14	50	55	7.5	7.9	7.3	7.5	26	6.4	"	+	—	
44	" 15									"	+	—	
45	" 16									"	+	—	
46	Aug. 17	60 mg. of uranium nitrate at 9 a.m.											
47	Aug. 18	62	67	7.7	7.8	7.4	7.8	28	6.4	Alkaline.	+	—	
48	" 19	56	59	7.6	7.8	7.4	7.8	20	6.3	"	+	+	
49	" 20									"	+	+	
50	" 21	54	61	7.5	7.8	7.3	7.7	19	6.6	"	+	+	
51	" 22									"	+	+	
52	" 23	58	62	7.5	7.8	7.3	7.6	18	6.3	"	+	+	
53	" 24	62	65	7.7	7.9	7.4	7.8	15	6.6	"	+	+	

TABLE V.

Dog 4. Weight 19 kilos.

First injection, 9 a.m., July 5, 1916.

July 3. 12 a.m. 19 gm. of sodium bicarbonate + 190 cc. of water.

From July 4 sodium bicarbonate + water was given every day, after bleeding.

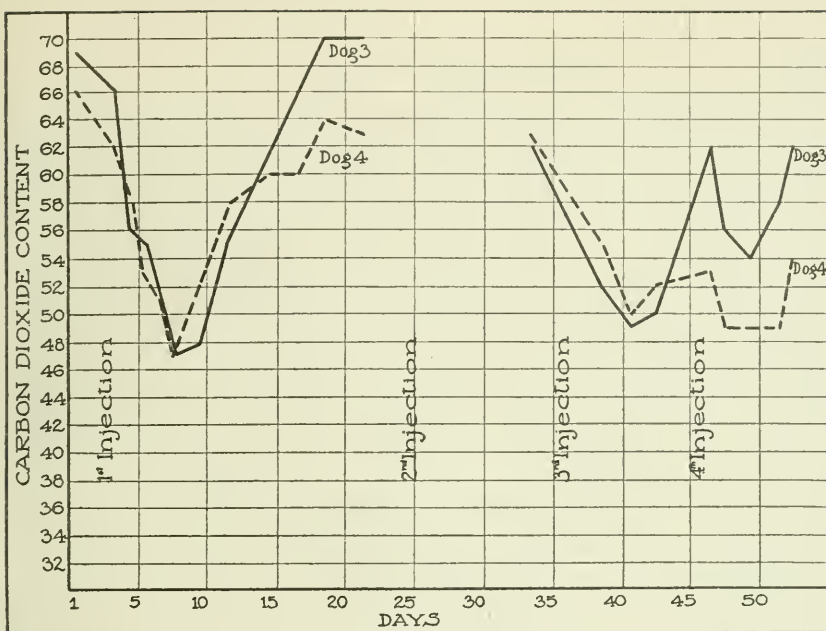
Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.			
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.	
				Direct.	A. B.	Direct.	A. B.						
Average.....		<i>per cent</i>	<i>per cent</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>log.</i>	<i>mg.</i>	<i>mg.</i>	Acid.	—	—	
1916													
1	July 3, 2 p.m.	66	70	7.8	8.0	7.5	8.0	13	6.0	Alkaline.	—	—	
2	“ 4												
3	July 5	29 mg. of uranium nitrate at 9 a.m.									Alkaline.	—	—
4	July 6	62	66	7.9	8.0	7.4	7.8	16	5.6	Alkaline.	Tr.	—	
5	“ 7	58	60	7.7	7.9	7.3	7.7	18	6.0	“	“	—	
6	“ 8	53	56	7.4	7.8	7.2	7.7	29	6.3	“	“	—	
7	“ 9									“	“	—	
8	“ 10	47	52	7.2	7.6	7.0	7.4	32	6.8	“	+	—	
9	“ 11									“	+	+	
10	“ 12	52	55	7.3	7.8	7.2	7.7	32	6.7	“	+	+	
11	“ 13									“	+	+	
12	“ 14	58	61	7.4	7.8	7.2	7.6	38	6.3	“	+	+	
13	“ 15									“	+	—	
14	“ 16									“	Tr.	—	
15	“ 17	60	65	7.8	8.0	7.5	7.9	28	6.3	“	“	—	
16	“ 18									“	“	—	
17	“ 19	60	66	7.8	7.9	7.4	7.9	36	6.1	“	“	—	
18	“ 20									“	“	—	
19	“ 21	64	68	7.9	8.0	7.4	7.8	26	5.8	“	“	—	
20	“ 22									“	“	—	
21	“ 23									“	“	—	
22	“ 24	63	66	7.8	8.0	7.4	7.8	25	5.8	“	“	—	
23	“ 25									“	“	—	
24	“ 26									“	“	—	
25	“ 27			8.0	8.1	7.4	7.7	20	6.0	“	“	—	
25	July 27	29 mg. of uranium nitrate at 9 a.m., after bleeding.											
26	July 28									Alkaline.	+	—	
27	“ 29			7.9	8.0	7.5	7.9	28	6.1	“	+	—	
28	“ 30									“	+	—	
29	“ 31									“	+	+	



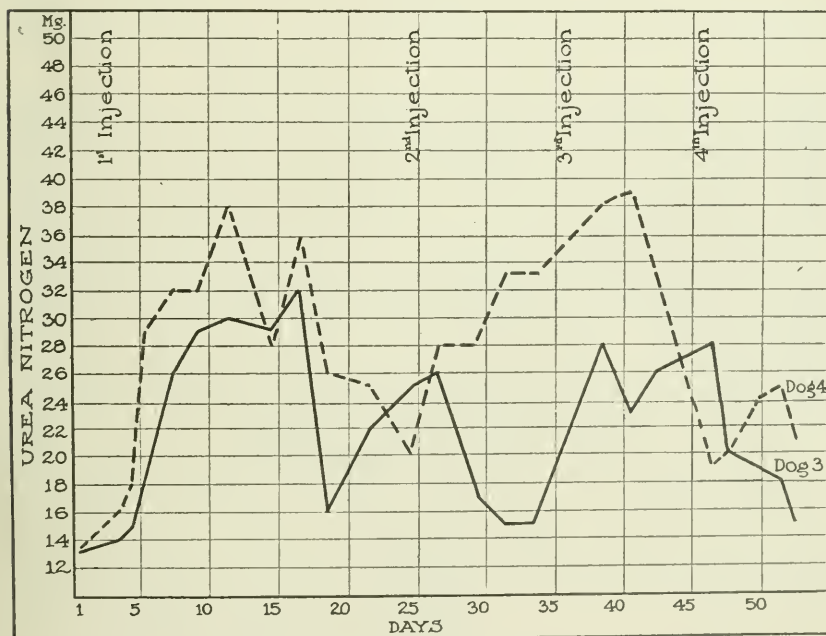
TABLE V—*Concluded.*

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.			
		Direct.	Funnel.	Serum.		Plasma.				Reaction.	Albumin.	Casts.	
				Direct.	A. B.	Direct.	A. B.						
30	Aug. 1	per cent	per cent	log.	log.	log.	log.	mg.	mg.	Alkaline.	+	+	
31	" 2			7.7	7.9	7.3	7.7	28	5.8	"	+	—	
32	" 3			7.8	7.9	7.4	7.8	33	6.3	"	+	—	
33	" 4									"	+	—	
34	" 5	63	65	7.7	7.8	7.4	7.7	33	6.1	"	Tr.	—	
35	" 6									"	"	—	
36	Aug. 7	36 mg. of uranium nitrate at 9 a.m.									Alkaline.	Tr.	—
37	Aug. 8									Alkaline.	Tr.	—	
38	" 9									"	+	—	
39	" 10	55	58	7.5	7.6	7.2	7.5	38	5.8	"	+	—	
40	" 11									"	+	—	
41	" 12	50	52	7.5	7.6	7.3	7.6	39	6.0	"	+	—	
42	" 13									"	+	—	
43	" 14	52	54	7.6	7.7	7.3	7.5	33	6.4	"	+	—	
44	" 15									"	+	—	
45	" 16									"	+	—	
46	Aug. 17	87 mg. of uranium nitrate at 9 a.m.									Alkaline.	+	—
47	Aug. 18	53	55	7.7	7.8	7.4	7.8	19	6.6	Alkaline.	+	—	
48	" 19	49	52	7.6	7.9	7.3	7.7	20	7.0	"	+	+	
49	" 20									"	+	+	
50	" 21	49	53	7.6	7.7	7.3	7.7	24	6.9	"	+	+	
51	" 22									"	+	+	
52	" 23	49	52	7.6	7.7	7.3	7.7	25	7.0	"	+	+	
53	" 24	54	58	7.7	7.8	7.4	7.7	21	7.0	"	+	+	

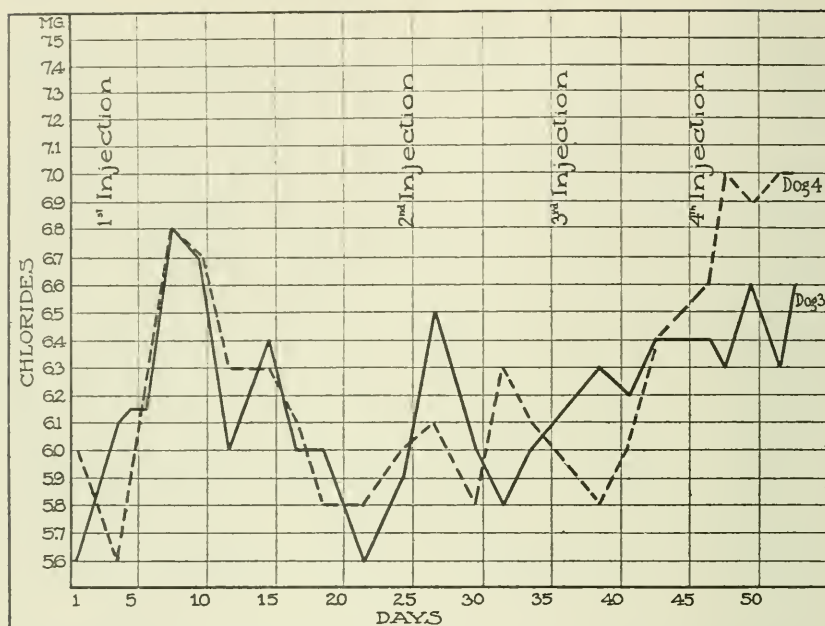
administration was commenced. The increase in the blood urea in the dogs receiving soda is comparable with that in the dogs receiving no soda except following the large fourth injection, when the dogs receiving soda showed a less pronounced rise. The increase in the plasma chlorides was consistently lower throughout the experiments in the dogs receiving the soda. This was especially marked following the fourth injection of uranium. The soda administered was sufficient to keep the urine in these dogs persistently



TEXT-FIG. 4. Curves showing the carbon dioxide content of the plasma of Dogs 3 and 4 after injections of uranium nitrate. Soda administered.



TEXT-FIG. 5. Curves showing the urea nitrogen in 1 cc. of blood in Dogs 3 and 4 after injections of uranium nitrate. Soda administered.



TEXT-FIG. 6. Curves showing the chlorides in 1 cc. of the plasma in Dogs 3 and 4 after injections of uranium nitrate. Soda administered.

alkaline. The albuminuria was persistent from the time of the first appearance, but was definitely less marked than in the animals receiving no soda. Casts were likewise less abundant in these animals and disappeared entirely for longer intervals of time. In general, following the fourth injection, the carbonate dogs showed a considerable difference from the control dogs as to the diminution of carbon dioxide content and as to increase of chlorides and urea in the blood.

The protocols of the histological examination of the kidneys follow.

*Dog 1. Weight 8 Kilos. No Soda.*

Day of experiment.	Absolute dose.	Uranium nitrate.	Relative dose per kilo.
		gm.	
1	0.012		1.5
22	0.012		1.5
33	0.015		1.85
44	0.036		4.5
52	Killed.		

Interval before fixation of tissues: a few minutes.

Under the low power the glomeruli appear normal. The epithelium of the proximal convoluted tubules appears swollen, especially in the tubules in the region of the corticomedullary junction. The epithelium of the ascending loops of Henle appear swollen. The medulla appears normal. The medullary rays stand out conspicuously.

Under high power some of the glomeruli exhibit moderate congestion, others appear normal. The epithelium of the proximal convoluted tubules is greatly swollen, but the nuclei are well preserved and only rarely is there any evidence of necrosis. The distal convoluted tubules are almost all normal, except for the presence of serum in the lumina of some. The medulla appears normal.

*Diagnosis.*—Marked cloudy swelling of the proximal convoluted tubules, especially those near the corticomedullary junction (Fig. 1).

*Dog 2. Weight 27 Kilos. No Soda.*

Day of experiment.	Uranium nitrate.	
	Absolute dose. <i>gm.</i>	Relative dose per kilo. <i>mg.</i>
1	0.040	1.5
22	0.040	1.5
33	0.050	1.85
44	0.120	4.5
52	Killed.	

Interval before fixation of tissues: a few minutes.

Under low power this kidney appears highly abnormal with moderate to marked congestion of the glomerular tufts and with patches of marked epithelial swelling in the proximal convoluted tubules, of interstitial edema, and of serum in the lumina in the cortex, especially in the corticomedullary region. The medulla exhibits marked congestion and edema with some swelling of epithelium and with serum or casts in the lumina.

Under high power congestion of the glomeruli is confirmed. The proximal convoluted tubules show intense epithelial swelling and in many places the nuclei are pale or necrotic. In many places the epithelium has entirely desquamated from these tubules, leaving simply the basement membrane. The distal convoluted tubules and ascending limbs of Henle are in many places encroached upon and compressed by interstitial edema and exhibit degeneration of epithelium and serum or casts in many of the lumina. In some of the epithelial cells of the distal convoluted tubules there is a yellowish pigment resembling hemosiderin.

*Diagnosis.*—Intense cloudy swelling and necrosis of the epithelium of the proximal convoluted tubules. Moderate cloudy swelling of the epithelium of the ascending limbs of Henle and the distal convoluted tubules. Interstitial edema of the cortex and medulla. Serum and casts in tubule lumina. Congestion of glomerular tufts (Fig. 2).

*Dog 3. Weight 13.5 Kilos. Soda.*

Day of experiment.	Uranium nitrate.	
	Absolute dose. <i>gm.</i>	Relative dose per kilo. <i>mg.</i>
1	0.020	1.5
23	0.020	1.5
34	0.025	1.85
44	0.060	4.5
52	Killed.	

Interval before fixation of tissues: a few minutes.

Under low power the picture is practically normal.

Under high power the glomeruli appear normal. The epithelium of all the tubules is in fair condition with little swelling, although this can be observed in occasional cells in the proximal convoluted tubules. The medullary cells are normal. The lumina are almost entirely free from serum.

*Diagnosis.*—Approximately normal.

*Dog 4. Weight 19 Kilos. Soda.*

Day of experiment.	Uranium nitrate.	
	Absolute dose. <i>gm.</i>	Relative dose per kilo. <i>mg.</i>
1	0.029	1.5
23	0.029	1.5
34	0.036	1.9
44	0.087	4.6
52	Killed.	

Interval before fixation of tissues: a few minutes.

Under low power the picture is normal except for serum or casts in the lumina of the medullary tubes.

Under high power the glomeruli seem normal. The proximal convoluted tubules show little cloudy swelling. The medulla is normal except for serum or casts in the lumina in a considerable number of the tubules.

*Diagnosis.*—Slight cloudy swelling. Serum or casts in the medullary tubules (Fig. 3).

It is a well established fact that uranium nitrate produces primarily and most conspicuously degeneration and necrosis of the epithelium of the renal tubules, and especially of the proximal convoluted tubules. The physiological studies of Schlayer and Hedinger (26), Pearce, Hill, and Eisenbrey (27), and others have shown that the reaction of the vascular apparatus of the kidney may be impaired or not by uranium nitrate, depending upon the dosage employed. Christian and O'Hare (28) have found that uranium nitrate causes also a lesion of the glomeruli characterized by the presence of hyaline droplets in the capillary loops and by other changes.

MacNider (29) reported a difference in the degree of pathological change in the kidney following uranium in dogs given sodium carbonate intravenously as compared with control dogs receiving no soda. The most marked difference was seen in the degree of involvement of the epithelium of the convoluted tubules.

It is evident from a study of the histology of the kidneys that the kidneys from both dogs receiving no soda exhibited more pronounced nephritis than those of either of the dogs receiving soda. The most severe nephritis occurred in Dog 2, which received the largest absolute dose of uranium; Dog 1, which received the smallest absolute dose of uranium, but no soda, showed also definitely more marked lesions than did Dogs 3 and 4, which received soda.

From the facts given both in the functional studies and in the pathological examination, it is clear that the nephritis in the dogs receiving sodium bicarbonate is less severe than that in the control dogs.

The toxic effect of uranium for the kidney is usually ascribed to the action of the metal as such. According to the experimental results of MacNider the toxicity of uranium runs parallel with its ability to lead to the formation of various acid bodies, and if the appearance of these substances in the urine is delayed and their amount in the body diminished by the administration of alkali, there is less evidence of the toxic action of the metal. In order to exclude the possibility of the toxic salt of uranium having been itself rendered inert by the direct action of sodium carbonate, he injected two animals with uranium nitrate in which the solvent for the uranium was a 3 per cent solution of sodium carbonate in 0.9 per cent sodium chloride. The toxic effect of uranium was in no way diminished when employed in a 3 per cent solution of the carbonate. He ascribes the protective action of sodium carbonate in uranium nephritis to the neutralization of acid bodies produced by the uranium in the animal economy.

In the study of the hydrogen ion concentration of the blood, the most consistent results were obtained by Marriott's modification of the technique, the figure obtained after aeration of the dialysate from the serum and given in the tables in the column "serum A. B." Determination upon the plasma by the same method gave, on the whole, parallel but probably less consistent results. A comparison of the methods of Marriott and of Van Slyke shows a greater delicacy in the Van Slyke method, so that while the evidences as to the acid



base equilibrium of the blood afforded by the two methods agree, Marriott's method is hardly delicate enough to permit of as satisfactory conclusions in such experiments as the present ones.

*Action of Alkali and of Acid upon the Carbon Dioxide Content in Plasma.*

Acid was administered by stomach tube to two dogs and alkali to two other dogs. For the acid, hydrochloric acid was chosen, and for the alkali, sodium bicarbonate. 1 cc. of 0.5 per cent hydrochloric acid per kilo of body weight was introduced into the stomach through the stomach tube. When acid was thus administered a diminution of the plasma carbon dioxide content developed, but since in this experiment a severe nephritis resulted, it might be questioned whether the decrease of the carbon dioxide content was produced wholly by the acid administered or in part also by the acidosis of the nephritis.

The microscopic examination gave clear evidence of nephritis in these kidneys.

When alkali (10 per cent sodium bicarbonate) was administered, the plasma carbon dioxide content of the blood showed a constant increase (Tables VI to XI.)

TABLE VI.

Dog 5. Weight 20.5 kilos.

Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	<i>per cent</i>	<i>per cent</i>				
Aug. 5	57	59	Acid.	—	—	
" 6	56	59	"	—	—	Acid at 10 a.m., after bleeding.
" 7	49	52	"	++	++	Died at 11 a.m.

TABLE VII.

Dog 6. Weight 18 kilos.

Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	<i>per cent</i>	<i>per cent</i>				
Aug. 5	58	60	Acid.	—	—	Acid at 10 a.m., after bleeding.
" 6	55	58	"	—	—	
" 7	48	53	"	++	++	

TABLE VIII.

Dog 7. Weight 10.5 kilos.

Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	<i>per cent</i>	<i>per cent</i>				
Aug. 5	56	58	Acid.	—	—	After bleeding, 200 cc. of 10 per cent sodium bicarbonate were given.
" 6	55	58	"	—	—	
" 7	60	63	Alkaline.	—	—	

TABLE IX.

Dog 8. Weight 14 kilos.

Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	<i>per cent</i>	<i>per cent</i>				
Aug. 5	56	58	Acid.	—	—	After bleeding, 280 cc. of 10 per cent sodium bicarbonate were given.
" 6	55	58	"	—	—	
" 7	60	63	Alkaline.	—	—	

TABLE X.

Dog 3. Weight 13.5 kilos.

	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
	<i>per cent</i>	<i>per cent</i>				
Average.....	56	63	Acid.	—	—	2 hrs. after 135 cc. of 10 per cent sodium bicarbonate were given.
	69	71	Alkaline.	—	—	

TABLE XI.

Dog 4. Weight 19 kilos.

	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
	<i>per cent</i>	<i>per cent</i>				
Average.....	58	64	Acid.	—	—	2 hrs. after 190 cc. of 10 per cent sodium bicarbonate were given.
	66	70	Alkaline.	—	—	

## DISCUSSION.

These investigations show that the nephritis produced by means of uranium nitrate presents a diminution of the plasma carbon dioxide content, associated with an increase of blood urea and plasma chlorides and the appearance of albumin and casts in the urine. These changes indicate the presence of an acidosis in the nephritis produced by uranium nitrate.

Moreover, both the nephritis thus produced and the acidosis which accompanies it can be diminished by means of sodium bicarbonate. In dogs receiving sodium bicarbonate and given uranium nephritis, there is maintained a higher plasma carbon dioxide content, a less pronounced increase of chlorides in the blood, as well as

a diminution of albumin and casts in the urine as compared with animals given uranium nephritis and receiving no soda. In severe nephritis the amount of urea is also diminished in the carbonate dogs as compared with the controls. The nephritis of the carbonate dogs is less severe as regards the histological picture than that of the controls.

#### CONCLUSIONS.

1. The presence of an acidosis in dogs with experimental uranium nephritis is demonstrable by the Van Slyke-Stillman-Cullen method and that of Marriott. It is detected more readily by the former method.

2. This acidosis is associated with increase in the blood urea and plasma chlorides and with the appearance of albumin and casts in the urine.

3. The oral administration of sodium bicarbonate diminishes the acidosis, the increase in plasma chlorides, the amount of albumin and casts in the urine, and, to a lesser degree, the increase in the blood urea following the administration of uranium. It also diminishes the severity of the changes produced by uranium in the kidneys.

4. The oral administration of sodium bicarbonate to normal dogs raises the carbon dioxide content of the plasma as determined by the Van Slyke-Stillman-Cullen method.

I wish to thank Dr. J. Harold Austin for his constant suggestions and interest throughout the course of this investigation, and Dr. Herbert Fox, Director of the William Pepper Clinical Laboratory, for extending to me the privileges of the laboratory.

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#### EXPLANATION OF PLATES.

##### PLATE 57.

FIG. 1. Dog 1. Section of the kidney of a dog with uranium nephritis, to which no soda was given.

FIG. 2. Dog 2. Section of the kidney of a dog with uranium nephritis, to which no soda was given.

##### PLATE 58.

FIG. 3. Dog 4. Section of the kidney of a dog with uranium nephritis, to which soda was given.







FIG. 1.

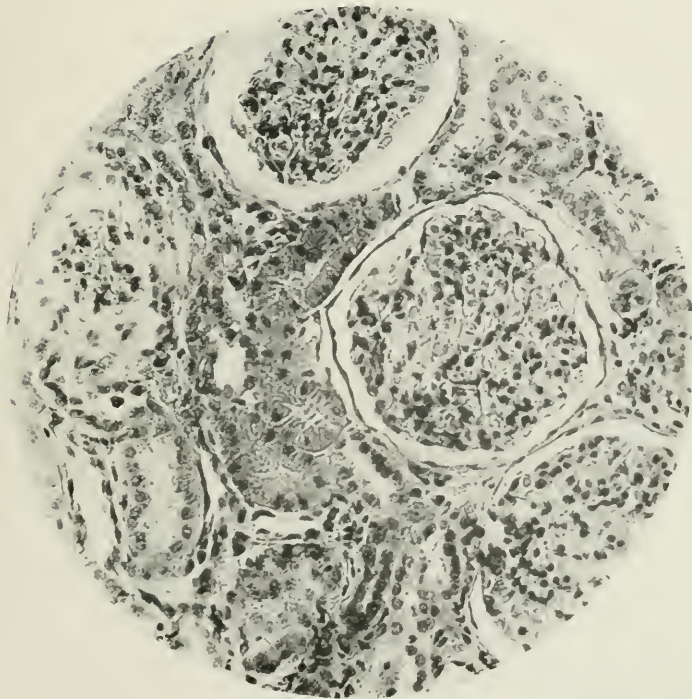


FIG. 2.

(Goto: Uranium Nephritis in the Dog )



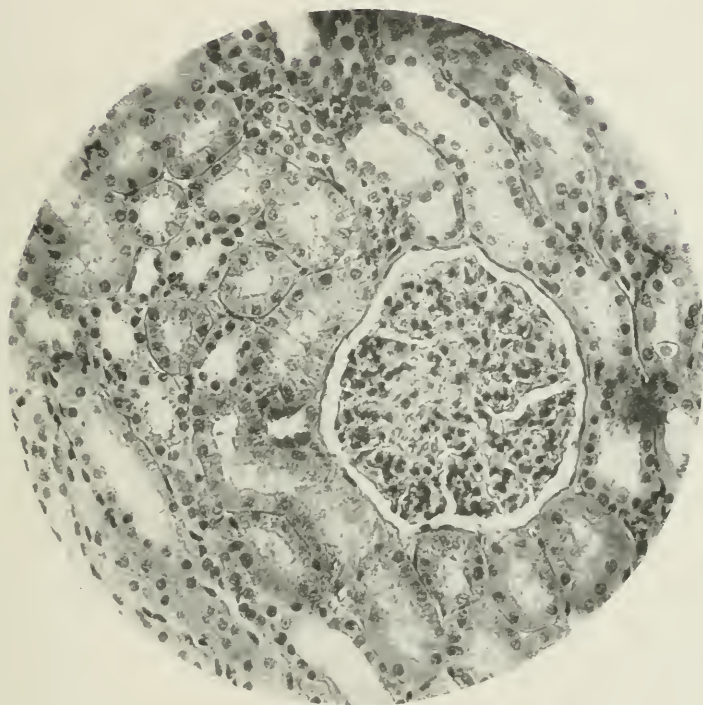


FIG. 3.

(Goto: Uranium Nephritis in the Dog.)



## CICATRIZATION OF WOUNDS.

### IV. MATHEMATICAL STUDY OF THE EXTRAPOLATION FORMULA AND OF THE CURVE OF CICATRIZATION.

By P. LECOMTE DU NOÛY.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and Hospital 21, Compiègne, France.)

(Received for publication, August 14, 1916.)

Several methods may be used to express the form of the curve of cicatrization by an equation, depending on whether the index  $i$  is or is not introduced into the calculation.<sup>1</sup> In such a curve, during the short time,  $dt$ , the area cicatrized,  $ds$ , remains proportional to the area,  $S$ , of the wound, which is expressed:

$$ds = Ks dt$$

If  $S_0$  is the initial area,  $K$  being a constant, integration gives:

$$T = \frac{1}{K} L \frac{S_0}{S}$$

We can now compute the values of  $\frac{1}{K}$ , corresponding to the observed numbers of  $S$  and  $T$ . These values increase regularly, and remain finite. Another factor must, therefore, intervene in the process of cicatrization. If this factor is the perimeter, and if we assume that  $K$  increases, the relation between  $K$  and the perimeter  $P$  is:

$$K = \frac{1}{K_1 + K'P}$$

If we assume that the wound does not alter its form as it cicatrizes,

$$\frac{P}{\sqrt{S}} = K''$$

<sup>1</sup> Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429. du Noüy, P. L., *ibid.*, 1916, xxiv, 451, 461.



from which, if  $K_2 = K' K''$

$$K = \frac{1}{K_1 + K_2 \sqrt{S}}$$

which gives the differential formula:

$$ds = \frac{1}{K_1 + K_2 \sqrt{S}} S dt$$

and by integration:

$$T = K_1 \text{Log}_e \frac{S_0}{S} + 2K_2 (\sqrt{S_0} - \sqrt{S})$$

This should represent the curve.<sup>2</sup>

Tables I and II show the results of a few calculations. In the equation the two coefficients  $K_1$  and  $K_2$  remain nearly constant for the same wound. This formula may therefore be considered as representative.

TABLE I.  
*Patient 263.*

Time (T)	Area (S)	Log S	Log $\frac{S_0}{S}$	$\sqrt{S}$	$\sqrt{S_0} - \sqrt{S}$	$T = 26 \log \frac{S_0}{S} + 2.6$ ( $\sqrt{S_0} - \sqrt{S}$ )
<i>days</i>	<i>sq. cm.</i>					
0	61.8	1.791		7.86		$K_1 = 26, K_2 = 1.3$
4	51.0	1.707	0.084	7.13	0.73	4.06
8	41.6	1.619	0.172	6.45	1.41	8.1
12	33.6	1.526	0.265	5.80	2.06	12.2
16	26.9	1.430	0.361	5.19	2.67	16.3
20	21.3	1.328	0.463	4.62	3.24	20.2
24	16.76	1.224	0.567	4.10	3.76	24.4
28	13.09	1.117	0.674	3.62	4.24	28.6
32	10.12	1.0051	0.786	3.18	4.68	32.5
36	7.77	0.890	0.901	2.79	5.07	36.6
40	5.94	0.774	1.017	2.44	5.42	40.5
44	4.5	0.653	1.138	2.12	5.74	44.2
48	3.4	0.531	1.260	1.84	6.02	48.3
52	2.53	0.403	1.388	1.59	6.27	52.0
56	1.88	0.274	1.517	1.37	6.49	56.4
60	1.38	0.140	1.651	1.175	6.685	60.3
64	1.01	0.004	1.787	1.005	6.855	64.2
68	0.74	̄1.869	1.922	0.860	7.00	68.1
72	0.54	̄1.732	2.059	0.735	7.13	72.0
76	0.4	̄1.602	2.189	0.633	7.23	75.6

<sup>2</sup> This calculation was made by Mr. de Rufz de Lavison.

TABLE II.  
*Patient 360.*

Time (T)	Area (S)	Log S	Log $\frac{S_0}{S}$	$\sqrt{S}$	$\sqrt{S_0} - \sqrt{S}$	$T = 25 \log \frac{S_0}{S} + 2.5$ ( $\sqrt{S_0} - \sqrt{S}$ )
<i>days</i>	<i>sq. cm.</i>					
0	113.1	2.053		10.6		
4	96.8	1.986	0.067	9.8	0.8	3.6
8	81.6	1.911	0.142	9.02	1.6	7.5
12	67.9	1.831	0.222	8.23	2.4	11.0
16	55.9	1.747	0.306	7.47	3.2	15.6
20	45.5	1.658	0.395	6.75	3.9	19.8
24	36.6	1.563	0.490	6.04	4.6	24.0
28	29.2	1.465	0.588	5.4	5.2	27.7
32	23.1	1.364	0.690	4.8	5.8	31.7
36	18.1	1.258	0.796	4.25	6.4	35.0
40	14.0	1.146	0.907	3.74	6.9	39.9
44	10.8	1.033	1.020	3.28	7.3	43.7
48	8.26	0.917	1.137	2.87	7.8	47.9
52	6.27	0.797	1.256	2.51	8.1	51.6
56	4.75	0.677	1.377	2.78	8.4	55.4
60	3.55	0.550	1.503	1.88	8.7	59.4
64	2.64	0.422	1.632	1.62	9.0	63.3
68	1.95	0.290	1.763	1.397	9.2	66.7
72	1.43	0.155	1.898	1.195	9.4	70.1
76	1.04	0.017	2.036	1.019	9.6	75.0
80	0.75	1.875	2.178	0.971	9.7	78.7

Other examples, without the details of calculation, are as follows:

*Patient 408.*

<i>t</i> observed.....	4.0	8.0	12.0	16.0	24.0	40.0	According to the above formula, $K_1=10$ , $2K_2=6$
<i>T</i> calculated.....	3.94	8.1	12.3	16.6	24.8	39.3	

*Patient 361.*

<i>t</i> observed.....	4.0	8.0	12.0	16.0	24.0
<i>T</i> calculated.....	3.4	8.2	12.0	16.1	24.1

*Patient 366.*

<i>t</i> observed.....	4.0	8.0	12.0	16.0
<i>T</i> calculated.....	4.1	8.1	12.0	16.01

*Corrected Formula for Narrow Wounds.*

The relation between the length and the width of the wound (the form) seems to play a more important part than the perimeter itself (the length of the outline). To express this in practical form, we have been searching for a formula adapted to long and narrow wounds. In wounds resulting, for instance, from longitudinal incision through muscles, an acceleration of the rate of cicatrization is observed within a few days before complete healing, the gain in time being sometimes 12 or 16 days. It is therefore necessary to introduce a new factor, the action of which was predicted by the calculation. This correction is not necessary in average wounds, because as stated above, the change of rate appears only when the length of the wound is very much greater than its width; in other words, when  $\frac{L}{l}$  ( $L$  being the length, and  $l$  the width) is such that:

$$10 < \frac{L}{l} < 25$$

In this case, as the perimeter is considerably increased in relation to the area, its action becomes then—but only then—important, and this explains why, in the case of ordinary circular or oval wounds, with a more or less hollowed outline, it is unnecessary to pay attention to the individual action of the perimeter.

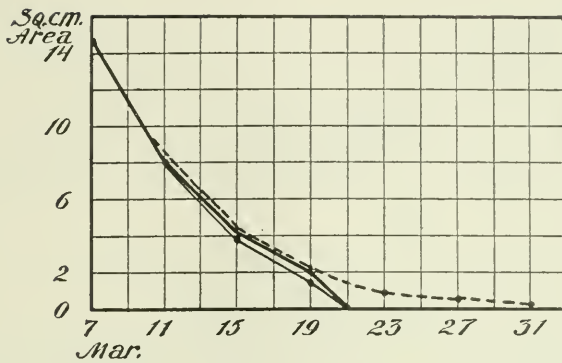
In very long wounds  $L$  is practically equal to  $\frac{P}{2}$  ( $P$  being the perimeter), and may be expressed by  $K'\sqrt{S}$ . The relation of the outline to the surface then becomes:

$$K' \frac{\sqrt{S}}{S}$$

Experiments showed that when  $K' = 1$ , this relation is practically equal to  $\frac{L}{l}$ , and the extrapolation formula becomes:

$$S'' = S'[1 - i(l' + \sqrt{T + l'})] - \frac{\sqrt{S'}}{S'}$$

The following examples show that this equation gives a far better approximation of the date of cicatrization of long and narrow wounds.



TEXT-FIG. 1. Patient 409. Wound of the leg.

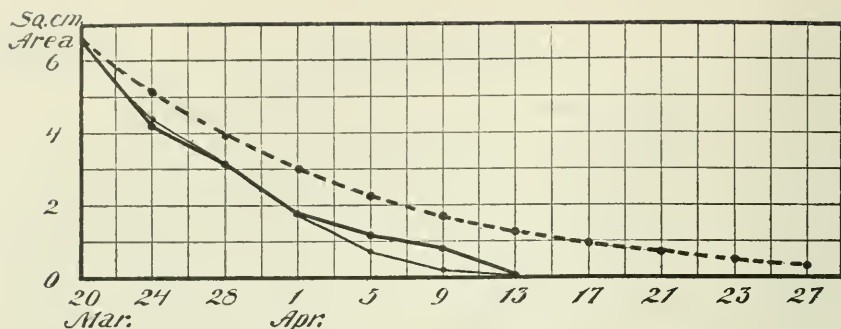
- observed curve.  
 - - - calculated curve, corrected.  
 ..... calculated curve, uncorrected.

Corrected according to  $S'' = S' [1 - i(t' + \sqrt{T + t'})] - \frac{\sqrt{S'}}{S'}$

Uncorrected according to  $S'' = S' [1 - i(t' + \sqrt{T + t'})]$

Patient 409 (Text-Fig. 1).

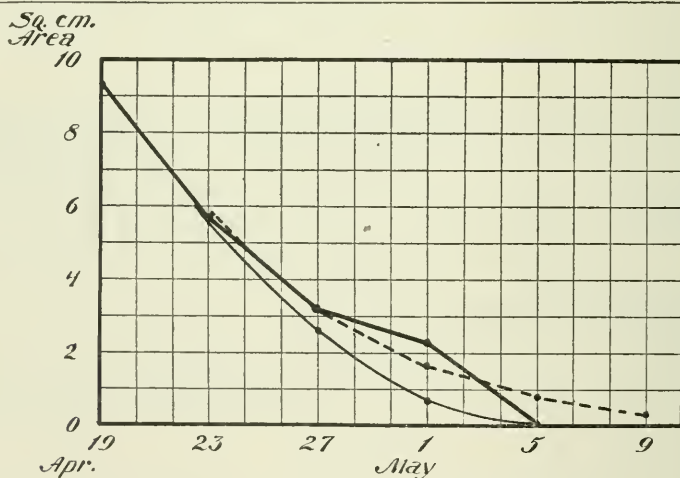
1916 Mar.	7	11	15	19	21	23	27	31
Observed area.....	14.6	8.2	4.3	2.0	0			
Calculated area (corrected).....		8.2	3.95	1.4	0			
“ “ (uncorrected)....		8.5	4.4	2.1		0.9	0.6	0.4
Length of wound (L) cm.....	13.2	12.3	10.6	7.4				
Width of wound (l) cm. maximum.....	1.6	1.0	0.6	0.3				
$\frac{L}{l}$ .....	8.2	12.3	17.6	24.6				



TEXT-FIG. 2. Patient 318. Wound of the leg. Normal rate until Mar. 20, 1916.

Patient 318 (Text-Fig. 2).

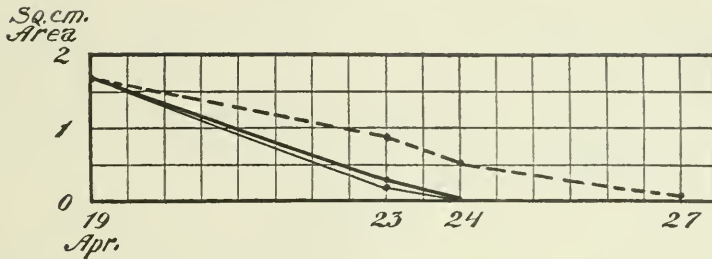
1916 Mar.	20	24	28	Apr. 1	5	9	13	17	21	25	27
Observed area.....	6.5	4.2	3.1	1.8	1.15	0.8	0				
Calculated area (corrected).....		4.3	3.1	1.8	0.7	0.2	0				
" " (uncorrected)...	6.6	5.1	3.9	3.0	2.25	1.7	1.28	0.95	0.70	0.50	0.37
$\frac{L}{l}$ .....	10.0	10.6	11.6	13.8	17.0	25.0					



TEXT-FIG. 3. Patient 415. Wound of the thigh.

Patient 415 (Text-Fig. 3).

1916 Apr.	19	23	27	May 1	5	9
Observed area.....	9.3	5.6	3.2	2.3	0	
Calculated area (corrected).....		5.5	2.6	0.72	0	
" " (uncorrected).....		5.7	3.2	1.65	0.78	0.35
$\frac{L}{l}$ .....	7.5	9.1	12.8	13.4		



TEXT-FIG. 4. Patient 415. Wound of the leg.

*Patient 415 (Text-Fig. 4).*

1916 Apr.	19	23	24	27
Observed area.....	1.7	0.3	0	
Calculated area (corrected).....		0.22	0	
“ “ (uncorrected).....		0.9	0.5	0.2
$\frac{L}{l}$ .....	9.0	12.5		

As the acceleration of the rate of cicatrization often occurs suddenly, the curves drawn by means of the above formula merely give a more exact determination of the date of complete healing. This formula goes to zero, which corresponds to the cicatrization.

As the perimeter becomes important only when the relation of  $\frac{L}{l}$  is above 10, the wound generally cicatrizes according to the uncorrected formula, until it suddenly begins to decrease in size. As soon as the relation between the two extreme dimensions (length and width) reaches a number between 10 and 20, the perimeter apparently becomes important, and reduces the time which should have been necessary for the cicatrization under ordinary circumstances. The fact that both epithelial borders are close to each other probably plays an effective part in this phenomenon.

The time taken is always 4 days.<sup>3</sup>

The corrected and uncorrected formulas can be simplified by expressing the calculated area of a wound after a certain period, as  $n \times t$  days. If  $t = 4$ ,  $nt = 4n$ .

<sup>3</sup> du Noüy, *J. Exp. Med.*, 1916, xxiv, 451.



If the first calculation is 1, the second 2, the third 3, and so on, up to the  $n^{-1}$  and the unknown  $n$ th; then the area  $S_n$ , at the end of  $n \times t$  days will be

$$S_n = S_{n-1} [1 - i (t + 1/\sqrt{nt})],$$

and the correction

$$- \frac{1/\sqrt{S_{n-1}}}{S_{n-1}}$$

or, if  $t = 4$ ,

$$S_n = S_{n-1} [1 - i (4 + \sqrt{4n})]$$

# THE DIGITALIZED DOG'S HEART AS AFFECTED BY AMYL NITRITE OR ATROPINE, STUDIED ELECTROCARDIOGRAPHICALLY.

By J. T. HALSEY M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

PLATES 59 TO 65.

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## INTRODUCTION.

This investigation was undertaken with the object of determining the action of amyl nitrite and of atropine on auriculoventricular heart block as produced by digitalis or allied substances. In order to do this it was essential first to work out a method of producing this block with a fair degree of regularity, and also to study the effects of amyl nitrite and of atropine on the normal electrocardiogram.

The accomplishment to a satisfactory degree of the first of these prerequisites was prevented by a number of factors. Chief of these was the tendency in normal dogs to sinus arrhythmia, resulting from the constantly changing vagus and sympathetic tone with its consequent sudden and frequent changes in the rate, rhythm, and conduction time, and the fact that in dogs central vagus stimulation often results in sinus inhibition or block, rather than in auriculoventricular block. As all the drugs used in this investigation exert direct or indirect stimulating or depressing actions on both the vagal and sympathetic control of the heart's function, this constantly occurring normal change in the activity of these two mechanisms proved disturbing and must always be considered in the interpretation of the various phenomena observed.

## *Methods.*

The observations are based on thirty-eight experiments in thirty-four animals. In almost all the experiments dogs were used. Three attempts were made to produce auriculoventricular block in cats,

but these were entirely unsuccessful, strophanthin, although slowly and cautiously given, causing none of the usual vagus effects. This failure probably occurred because the cats were restless throughout the experiment, so that the accelerator mechanism from the start maintained complete control of the circulation (Straub (1)).<sup>1</sup>

In the earlier experiments, after taking control curves, light ether anesthesia was induced, a cannula was inserted into the femoral vein, and after waiting until the electrocardiogram appeared to have returned to its original form, the various solutions were injected and curves taken at short intervals. In later experiments the exposure of the vein was made under cocaine-adrenalin anesthesia and no ether was used. This method appeared to possess certain advantages, among which may be mentioned the fact that it was thus possible to avoid the alteration of the normal rate and rhythm of the heart, which ether always causes, and which frequently persisted for an hour or more after cessation of its administration. Control observations showed that in the dosage employed (0.5 to 1 cc. of cocaine, 1 : 400, and of adrenalin chloride, 1 : 30,000) these drugs produced no detectable alteration in the electrocardiogram. The blood pressure in a number of experiments was recorded by means of a Harvard membrane manometer inserted into the femoral artery.

In a few experiments, in order to keep the animals still, so that sudden and irregular alterations in the circulation might be avoided, a light but persistent narcosis by the administration of luminal sodium was induced.

In doses of 0.04 to 0.06 gm. per kilo by mouth or subcutaneously this drug usually caused a quiet drowsy or stuporous condition, persisting for 24 hours or longer. At times, however, it caused a marked lowering of the blood pressure and persistent rapid pulse and appeared, in some experiments, to prevent the development of the usual digitalis actions. Its use was, therefore, abandoned. The usual effects of its administration were an increase in the heart rate and an abolition of the sinus arrhythmia which is almost invariably present in the quiet dog. After 36 to 48 hours these effects passed off and the heart returned to its original action. No effects on the electrocardiogram were noted other than were due to the above, which would naturally result from a diminution of the vagus tone, such as is to be expected from large doses of any of the lipoid-soluble narcotics.

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<sup>1</sup> Straub (1) reports similar results with these animals.

It is important to report that in four of the thirty-four dogs used in these experiments a spontaneous auriculoventricular block was observed. At The Rockefeller Institute for Medical Research in the past 6 years the same phenomenon has been observed in at least four additional cases. The occurrence of this condition, by no means rare, should be remembered by those using these animals, and should lead to caution in interpreting the significance of its appearance. An example of this is seen in Fig. 1, A (Experiment 85).

All observations were made with Lead II. As a majority of the animals survived, control curves were in many instances taken on the following day.

*The Effects of Strophanthin and Digitalis on the Normal Dog's Heart.*

*Strophanthin.*

*Dosage.*—Crystalline strophanthin was injected into the femoral vein in amounts ranging from 20 to 95 per cent of the average lethal dose (1.01 mg. per kilo, according to Jamieson (2)), using a solution of which 1 cc. contained 0.02 mg. of Thoms crystalline *g*-strophanthin. In the majority of the experiments from 30 to 40 per cent of the lethal dose was injected in about 15 minutes. It was found that the dose which produced a marked vagus effect, as shown by pronounced slowing, auriculovenricular block, or exaggerated sinus arrhythmia, usually lay between 30 and 40 per cent of the lethal dose. Doses above 40 per cent of the lethal dose were likely to produce ectopic ventricular beats or acceleration of the sinus rate, or both. As in many of the experiments the observations extended over periods of several hours and as the various strophanthin effects pass off more reness rapidly, these effects were maintained in a number of experiments by intermittent slow injection of very dilute solutions. The majority of the animals recovered promptly, often within a few hours, from doses up to 60 per cent of the minimum lethal dose.

*Rate (Excluding Changes Due to Ectopic Contractions).*—In twelve of the nineteen experiments the administration of strophanthin was followed by slowing (Table I, Group A), in four by slowing followed by acceleration (Table I, Group B), and in three by acceleration without previous or subsequent slowing (Table I, Group C).

In interpreting the significance of acceleration of the sinus rate, it is impossible to exclude the influence of psychic factors, but in this series as well as in the digitalis series, reported below, there were a number of instances in which, as far as could be determined by close observation of the dog, the acceleration did not appear to be preceded or accompanied by any evidence of excitement or unrest. The acceleration noted is consequently attributed to a direct action on the sinus node, such as was observed by Rothberger and Winterberg (3) in their experiments on dogs in which the cardiac nerves had been cut.<sup>2</sup>

*Rhythm.*—The initial rhythm was affected as follows: auriculo-ventricular block resulted in nine of nineteen experiments. In one of these (No. 52) it was present in the control curve, was abolished by amyl nitrite, and returned for a short time after strophanthin had been injected. In only two experiments, Nos. 56 (Fig. 2, B) and 57, were fairly persistent auriculoventricular blocks obtained, but in six others occasional blocks were produced.<sup>3</sup> In nearly all these cases the sinus arrhythmia was markedly exaggerated by strophanthin. Occasional sinus block was produced in two experiments with interauricular (P-P) intervals ranging up to 1.57 seconds. The sinus arrhythmia was exaggerated by strophanthin in fourteen experiments, but was little or not at all affected in three, and was lessened in two experiments.

*Auriculoventricular (P-R) Interval.*—This was variously affected by the administration of strophanthin. At some time in the majority of the experiments (fourteen out of nineteen), it was more or less lengthened, on the average by 17 per cent of the initial conduction time. At times this effect was very pronounced, as for example in Experiments 56, 59, and 63, where it was increased from averages of 0.118, 0.112, and 0.116 before, to averages of 0.152, 0.145, and 0.146

<sup>2</sup> Under the conditions in my series of experiments a direct stimulation of the central accelerator mechanism may also occur and is not impossible in view of the known stimulation of another closely related sympathetic (the vasomotor) center.

<sup>3</sup> In the endeavor to secure the desired auriculoventricular block, the rate of injection, the size of the dose, and the concentration of the solutions injected were varied in different instances, but without success.

TABLE I.

*Effect of Strophanthin on Sinus Rate in Dogs.*

Group.	No. of experiment.	Weight.	Maximum effect on rate.		Lethal dose given.	Duration of administration.	Remarks.
			Before.	After.			
		<i>gm.</i>			<i>per cent</i>	<i>min.</i>	
A	52	5,000	180	140	40	14	A-V block.
	54	7,500	110	76	60	116	Pronounced S-A.
	56	6,250	140	84	40	13	A-V block.
	57	6,900	109	65	35.7	17	Sinus block preceded by A-V block.
	58	6,250	120	54	54	164	Sinus block.
	60	6,000	80	65	35	12½	Ectopic beats before and after strophanthin.
	62	8,800	80	72	38	180	
	63	6,600	120	96	30	13	
	64	7,750	92	60	40	55	
	65	8,250	162	96	37	56	Dog had had atropine 87 min. before rate of 162.
	67	8,600	140	80	37	65	Dog had had atropine 60 min. before rate of 140.
	69	11,800	70	60	35	29	Second strophanthin injection started 205 min. after first strophanthin injection and 140 min. after atropine.
			120	60	Additional 20	20	
B	59	7,000	110	100	25	5	This dog was restless.
				235	40	10	A few ectopic beats occurred (7 ectopic to 50 nomotopic) when this rate was reached.
	61	8,500	120	90	24	7	The acceleration in this experiment occurred so late (24 min. after the dose of 35 per cent) that it may not have been due to strophanthin.
				190	35	15	
	71	10,000	145	130	25	9	
				155	30	13	
C	73	6,700	130	105	34	14	
				145	55	42	
	55	8,000	102	235	50	16	Following the strophanthin ectopic beats persisted most of the time for 100 min., but the rate of 235 is without ectopic beats.
	68	7,200	150	240	30	12	Atropine 135 min. earlier.
	72	9,750	140	240	56	30	

The apparent discrepancy as to frequency of A-V blocks between the table and the text (page 732) is due to the fact that A-V blocks are mentioned under "remarks" only when present at, or near the time when minimum figures occurred.



seconds after the administration of strophanthin. In the three experiments the longest intervals before giving strophanthin were 0.14, 0.14, and 0.13 seconds; after it they were 0.19, 0.165, and 0.21 seconds. In Experiment 62 the longest P-R interval in the control curves was 0.18 second, but after strophanthin there was one of 0.37 second. In two experiments the P-R interval was first lengthened, then shortened; in two others first shortened then lengthened; and in one it was never lengthened, but was slightly shortened from an average of 0.10 to 0.094 second, the sinus rate varying only from 92 to 95 per minute.

In many instances the change in conduction time was obviously influenced by the change in sinus rate, and in many others the changes noted were slight. But there is no doubt that as a general rule the P-R time was lengthened by strophanthin. Pronounced shortening of the conduction time was, however, observed at times in experiments in which it could not be attributed to change in the sinus rate, but would appear to have been due to sympathetic stimulation, either central, from psychic cause, or as a result of direct drug action, or to drug action on the conduction paths themselves.<sup>4</sup> An example of this is Experiment 64 in which the P-R time originally varied from 0.09 to 0.11 second, but after a dose of strophanthin it ranged from 0.10 to 0.17 second, and then after a second dose ranged between 0.07 and 0.10 second, the sinus rate varying only from 80 to 78 to 82 per minute.<sup>5</sup>

*P Wave.*—In the majority of the experiments (fourteen out of nineteen) the P wave showed no significant changes. In the five others the administration of strophanthin was followed at certain stages by a lessening of the height of this wave, which varied from strongly positive to isoelectric and occasionally became diphasic. In one of these experiments occasional negative P waves were noted. As the height of the P waves varies spontaneously in dogs from time to time and may even be negative in control curves, only a distinct increase or decrease has been considered to be significant. A lessen-

<sup>4</sup> For the latter mode of action these experiments produce no evidence, nor is it known to me that an analogous action is exerted by any other drug.

<sup>5</sup> The quiet behavior of this dog and the lack of increase in the sinus rate speak strongly against psychic stimulation as a cause of this effect on the P-R time.

ing of its height (Einthoven, and Einthoven and de Waart (4))<sup>6</sup> or a change to negative appears to result under certain conditions from increased vagus tone, but there are of course other possible explanations for this change in the wave (Rothberger and Winterberg (5)).<sup>7</sup>

*Ventricular Complex (R and T Waves).*—This frequently showed changes (in sixteen out of nineteen experiments). Ectopic ventricular contractions, usually of the left ventricular type, appeared after as little as 19, 25, 30, 31, and 33 per cent of the lethal dose, and almost invariably occurred if more than 40 per cent of the lethal dose was given in about 15 minutes. After the larger doses these ectopic beats were of many types. While nomotopic cycles persisted, the T wave was commonly altered by even smaller doses. Slowing of the sinus rate appeared to exert some influence in favoring the occurrence of ectopic beats, while acceleration appeared to hinder it. In certain experiments large amounts (*e.g.*, Experiment 73, where 70 per cent of the minimum lethal dose was given in 67 minutes) failed to cause ectopic contractions.

As the effective vagus stimulating dose in dogs appears to lie between 20 and 40 per cent of the lethal dose, it is a matter of interest and perhaps of some importance that ectopic ventricular beats are so frequently produced by these doses. In Experiment 56 after 40 per cent of the lethal dose had been given in 13 minutes auriculo-ventricular block appeared (Fig. 2, B). The ventricular contractions were ectopic in origin, possibly as the result of the stimulation of tertiary centers by strophanthin, which had been previously injected. In all the experiments in which ectopic contractions followed the administration of strophanthin, they appeared during or shortly following its injection, with the exception of certain experiments which will be discussed below, and in which their more tardy appearance can be attributed to the action of amyl nitrite or atropine. In Figs. 3, D, 4 B, D, and E, 5, B, C, D, and E, and 6, G the various types of ectopic complexes noted may be seen.

<sup>6</sup> This was observed by Einthoven and de Waart (4) after stimulation of the vagus.

<sup>7</sup> For example, Rothberger and Winterberg (5) obtained negative P waves by simultaneous stimulation of the vagus and accelerator nerves and at times by stimulation of the accelerator alone.

The R wave, as long as nomotopic cycles were present, as a rule underwent no significant alterations in shape. In a few experiments it became much shorter.

The T wave changes were difficult to analyze, as this wave is variable in the normal controls and as it changes rapidly and markedly with changes in rate and rhythm. The change noted by Cohn, Fraser, and Jamieson (6) as characteristic for digitalis in human beings, *i.e.*, a change from one sign to the other, appeared in the majority of the cases where vagus effects (slowing, increase in sinus arrhythmia, etc.) occurred, and was also often present before these effects were apparent. On the other hand, marked vagus effects were at times apparent without the characteristic effect on the T wave. Frequently, especially where there was acceleration of the heart rate with or without the occasional occurrence of ectopic contractions the T wave became decidedly taller (Fig. 7, B), an alteration observed by a number of authors (Selenin (7), Nicolai and Simons (8), Straub (1), and Rothberger and Winterberg, and Einthoven and de Waart (9)), and considered, apparently correctly, by the four last named as the result of a direct stimulation of the sympathetic nerve structures (tertiary centers) in the ventricles.<sup>8</sup>

#### *Digitalis.*

The infrequency with which strophanthin produced persistent auriculoventricular block suggested the use of the entire digitalis body instead of the single active principle. For this reason digipuratum was administered in eleven experiments and a fluid extract of digitalis (Parke, Davis and Company) was used in three others. It is customary in therapeutics, no doubt with justice, to regard strophanthin as more easily absorbed by the heart muscle and its action as more prompt, but at the same time more transitory, than that of digitalis. It was therefore presumed that when digitalis was administered intravenously to dogs, the alteration of rhythm would come on more slowly and more uniformly and would be more

<sup>8</sup> In my experiments a direct stimulating effect on the accelerator (or sympathetic) centers may be involved, but Rothberger and Winterberg (9) observed identical changes following the administration of strophanthin in dogs after bilateral vagotomy and destruction of the stellate ganglia.

persistent than had been the case with strophanthin. The use of these bodies was, however, attended by no greater success than when strophanthin was used. Contrary to expectation, the effects produced by digitalis<sup>9</sup> ensued as promptly and passed off as rapidly as had been the case with strophanthin. The digitalized heart also reacted to amyl nitrite and to atropine just as did the strophanthinized heart. Apparently in the dog digitalis is rapidly excreted or in some other way rendered inactive. These observations are in accord with the fact that these animals are highly tolerant of digitalis by mouth and with the experience that it has been difficult to produce in them the ordinary digitalis effects by oral administration. Briefly summarized, the effects usually produced by digitalis or strophanthin under the conditions obtaining in this series were as follows: slowing of the sinus rate, increased conduction time, occasional auriculoventricular blocks, or exaggerated sinus arrhythmia. The T wave in many experiments became negative, but often was unaltered or became more positive.

Ectopic ventricular beats of the left ventricular type frequently followed the administration of doses not exceeding 40 per cent of the minimum lethal dose. Doses larger than this usually caused the occurrence of ectopic ventricular contractions, which at first were of the left ventricular type but later were of various sorts.

*The Effects of Amyl Nitrite and of Atropine on the Normal and Digitalized Dog's Heart.*

*Amyl Nitrite.*

Amyl nitrite has been employed clinically (Josué and Godlewski (10), Petzetakis (11), and Belloir and Dubos (12)) as a means of determining whether an auriculoventricular block is due to central vagus stimulation or to direct action on, or organic changes in the conduction paths. Its power of abolishing heart block or of accelerating the sinus rate, according to our present knowledge of its pharmacology, results from its ability to cause a fall of the blood pressure, which in turn depresses the vagus and stimulates the accelerator center.

<sup>9</sup> Twelve frog units per kilo were taken as the minimum lethal dose.

Earlier investigators of this drug concluded that it had a marked early stimulation effect on two of the important medullary centers, the vagus<sup>10</sup> and the respiratory, the action on the vagus being attributed by them to reflexes from the upper air passages (Filehne (13) and Brunton (14)). As it has been shown by Pilcher and Sollmann (15) that a third medullary center, the vasomotor, is stimulated even when these reflexes are excluded and as its similar action on the respiratory center is universally recognized, it is possible that the vagus center is also acted on in the same way; *i.e.*, direct stimulation.

The depression of vagus tone which is subsequently seen is due to the fall in blood pressure which amyl nitrite causes and which removes the physiological stimulus for vagus activity. This conception of the cause of the changes in vagus tone during the inhalation of amyl nitrite is in accord with the phenomena observed in this series and accounts for the sequence of events about to be described.

*Rate.*—In normal, strophanthized, and digitalized dogs the rate was often slowed at first by amyl nitrite, at times strikingly; as for example in Experiment 53 (Fig. 8, B), in which, after inhalation for 70 seconds, the rate fell from 66 to 42, and Experiment 64 where after inhalation for 20 seconds the rate fell from 72 to 56. This slowing occurred only in dogs in which the vagus was already exerting a marked effect, as evidenced by the slow rate, or auriculo-ventricular block, or sinus arrhythmia. As a rule, with continued inhalation the rate was markedly increased. In Experiment 53, for instance, the rate rose later to 195 per minute (Fig. 8, C).

The primary slowing is undoubtedly due to central vagus stimulation, direct or indirect. It was followed by acceleration, provided

<sup>10</sup> Filehne (13), for instance, reports an experiment in which the inhalation of amyl nitrite by a rabbit caused a fall in the heart rate from fifty-three to ten beats per 15 seconds. Pharmacologists have more recently apparently overlooked this early vagus stimulating effect, probably because in ordinary laboratory demonstrations the drug is inhaled through a tracheal cannula. This method, of course, minimizes reflex respiratory effects, while the anesthesia universally employed renders the vagus center less sensitive to stimulation either direct or indirect. I have occasionally seen a primary slowing of the heart rate of slight degree and short duration from amyl nitrite, even in tracheotomized and anesthetized animals. As this investigation was undertaken in the hope of obtaining information bearing on the clinical use of this drug in studying heart block and other arrhythmias, no experiments were done in which the drug was administered by tracheal cannula. Consequently in the majority of the observations made the effects of this vagus stimulation were very apparent.



enough amyl nitrite was given to cause vasodilation sufficient to affect the blood pressure, as was seen in all the observations in which the blood pressure was recorded. The curves show the reciprocal relation between the rise and fall in the blood pressure and the increase or diminution of vagus tone as indicated by the heart rate. These relations may be seen in Fig. 1, B to G. In several experiments where the blood pressure was not recorded even prolonged inhalation of amyl nitrite failed to cause acceleration of the heart rate, but the numerous records where the blood pressure was recorded show no instance where, in spite of a fall in the blood pressure, the heart rate was not accelerated markedly.

*Rhythm.*—As a rule, the rhythm was promptly and decidedly affected by the inhalation of amyl nitrite, sinus arrhythmia or auriculoventricular blocks, whether spontaneous or due to drugs, being (if the drug was given in sufficient doses) abolished or at least markedly lessened (Figs. 1, A, B, D, E, and G, and 8, B and C). In a number of the experiments, however, the effects were preceded by an exaggeration of the arrhythmia due to the vagus stimulation already referred to, and in a number of instances inhalation lasting for several minutes failed completely to abolish the arrhythmia or blocks. An illustration of this early vagus stimulation is seen in Fig. 8, B.

Especially striking was the vagus stimulation in Experiment 53 (Fig. 8, B) in which the interventricular (R-R) interval, which had previously ranged from 0.54 to 1.34 seconds, varied from 0.78 to 4.02 seconds during an inhalation lasting 70 seconds. In this experiment the sinus arrhythmia was not abolished until amyl nitrite had been inhaled for 7 minutes and 40 seconds. In Experiment 64 sinus block (or pronounced sinus arrhythmia) which occurred after strophanthin was given was not abolished by an inhalation lasting 140 seconds, but was abolished later by one lasting 20 seconds. In Experiment 52 a spontaneous auriculoventricular block was not abolished by the first inhalation lasting 60 seconds but was abolished by a second one lasting 90 seconds. In Experiment 56 amyl nitrite inhalation twice failed to abolish an auriculoventricular block due to strophanthin, although the inhalation was continued the second time for as long as 5 minutes. Unfortunately in all the observations cited above the blood pressure was not recorded.

In a number of other experiments in which the blood pressure was recorded, similar but less extreme vagus stimulation occurred, but later gave way to vagus depression (and probably central accelerator stimulation as well) when the vasodilating effect of amyl nitrite became sufficiently pronounced. Fig. 1, A to G



illustrates these effects, both before and after the administration of digitalis to a dog in which there was a spontaneous auriculoventricular block.

In view of the fact that whenever amyl nitrite produced sufficient vasodilation, as seen in the blood pressure records, it also invariably abolished or lessened the arrhythmia or blocks, it may be concluded that its failure to relieve the irregularity in a number of the earlier experiments was due to insufficient dosage.

*P-R Interval.*—The P-R interval was markedly altered by amyl nitrite. As a rule, the first effect during the stage of vagus stimulation was to lengthen the interval, but later, when a sufficient amount of the drug had been inhaled, the conduction time was markedly lessened, often to 50 to 60 per cent of its original length, while at the same time acceleration of the heart rate took place. These changes are well shown in Experiment 62 in which the P-R time originally varied from 0.09 to 0.16 second. After the inhalation of amyl nitrite for 15 seconds it ranged from 0.11 to 0.56 second, and after 60 seconds was shortened to a uniform length of 0.08 second.

*P Wave.*—The P wave was frequently markedly altered by amyl nitrite, usually becoming more positive after its inhalation (Figs. 6, B and C, and 7, B, C, and D). In Experiment 62 in which after the administration of strophanthin this wave varied from an isoelectric to a positive form, all P waves became strongly positive during the inhalation of amyl nitrite (Fig. 9, B, C, and D). This change in the P wave corresponds to the change in the form of this wave under the influence of sympathetic stimulation as described by Rothberger and Winterberg (5).

*Ventricular Complex.*—The ventricular complex, as long as it represented a normally contracting ventricle, often showed significant changes in the T but not in the R wave after amyl nitrite inhalation. The former was frequently altered by amyl nitrite, generally as follows: During the stage of vagus stimulation it often became less positive, but later, as a rule, when the vagus control had been abolished by this drug the T wave became markedly positive (Fig. 7, B, C, and D). When digitalis or strophanthin in not too large amounts had caused the appearance of ectopic beats, amyl nitrite almost always caused their temporary or permanent disappearance (Fig. 4, C). Even after very large doses of strophanthin or

digitalis had caused a constant succession of ectopic beats, the administration of amyl nitrite usually was followed by the reappearance of occasional normal cycles or by striking alteration in the form of the ventricular complexes so that they more closely resembled that of nomotopic ventricular contractions (Fig. 5, C).

In a single experiment (No. 84), in which 33 per cent of the lethal dose of digipuratum had been given 28 minutes earlier, ectopic ventricular contractions appeared for the first time 2 minutes after an inhalation of amyl nitrite, lasting 90 seconds, had abolished a pronounced sinus arrhythmia, and caused a marked acceleration of the sinus rate. The occurrence of these contractions, originating in the tertiary centers, stimulated as they were by the digipuratum previously given, may have been due to the abolition by amyl nitrite of the vagus tone by removing its negative inotropic action on the ventricle alone, or in combination with stimulation of the accelerator center. This explanation seems improbable in view of the fact that the ectopic beats continued to appear from time to time for 26 minutes after the amyl nitrite had been stopped, and that in many other observations<sup>11</sup> amyl nitrite exhibited a striking power of abolishing ectopic contractions if they were already occurring. On the other hand, in no other experiment did ectopic contractions develop so long after the administration of digitalis or strophanthin. Another factor to be considered in estimating the significance of this isolated observation is the occasional, if rare, spontaneous occurrence in dogs of ectopic ventricular contractions.<sup>12</sup>

In view of the similar reaction of arterial and heart muscle, and of the neuromuscular (intermediate) substance contained therein, to various pharmacological agents (*e.g.*, epinephrin) and physiological stimuli (*e.g.*, stimulation of sympathetic nerve supply), the effects observed in these experiments should have been expected. Because of the close relation of these structures and the known dominant action of amyl nitrite, that is, depression of the sympathetic neuromuscular organs in the arterial walls, it is not surprising that this drug produces a similar depressing action on the cardiac muscle through its neuromuscular (intermediate) substance. It is im-

<sup>11</sup> Later in the same experiment, when 44 per cent of the lethal dose of digipuratum had caused almost constant ectopic contractions, inhalation of amyl nitrite for 120 seconds caused them to disappear temporarily.

<sup>12</sup> In the series of thirty-eight experiments, this was observed but once, and then in a period when the heart rate was slow, which was not the case in this experiment.

portant to emphasize the fact that, in order to produce this depressing action on the tertiary cardiac centers, very large doses were necessary. Otherwise one might be tempted to expect that one could produce analogous effects in human patients by administering amyl nitrite. That this is possible cannot be denied, but one attempt to do so was unsuccessful.<sup>13</sup>

### *Atropine.*

Atropine also has been used by a large number of clinicians in the study of various types of cardiac irregularity, especially in cases of heart block, to determine whether the block is of vagal or other origin, but as far as is known to the writer it has not been studied experimentally under such conditions as obtain in this series. It is for this reason that it appeared desirable to study its effect on the normal and digitalized heart and to compare its action with that of amyl nitrite.

Atropine sulfate was administered intravenously to twelve dogs in doses ranging from 0.004 to 0.07 mg. per kilo. The effects on rate, rhythm, etc., varied markedly with the size of the dose. Small doses often produced the effects of central vagus stimulation; *i.e.*, lengthening of the auriculoventricular interval and auriculoventricular block. The sinus rate, if affected at all, was accelerated. Larger doses usually produced similar results at the start, but were followed quickly by the effects of vagus depression; namely, acceleration of the rate, shortened P-R interval, and abolition of sinus arrhythmia or of auriculoventricular block.

*Sinus Rate.*—The sinus rate was increased by atropine in each observation in which any change was noted, but in several experiments the smaller doses produced little or no effect. In no case did the repetition or increase in the size of the dose fail to cause marked acceleration of the sinus rate. The general effect of the action on sinus rate is seen in Table II. In the experiments in which the heart was slowed by previous administration of strophanthin or digitalis the rate after atropinization was increased to approximately

<sup>13</sup> This was in a patient with an attack of paroxysmal tachycardia of auricular origin, seen by me through the courtesy of Dr. Lerch in his service at the Charity Hospital in New Orleans.

the same figure as had been reached after similar doses given before strophanthin or digitalis had been administered; that is, the increase in rate was as great after as before digitalization. In the majority of cases, however, the acceleration produced by atropine did not last as long in strophanthinized or digitalized dogs as in the controls.

*Rhythm.*—The effects on rhythm in twenty-four observations varied with the size of the dose. A small dose usually caused auriculo-ventricular block, which was abolished by repetition of the dose (Fig. 10, B and C), or if a block was already present, it was first exaggerated and then abolished. For example, in Experiment 62 administration of strophanthin was followed by marked sinus arrhythmia and occasional auriculoventricular blocks. A first injection of 0.016 mg. of atropine sulfate per kilo caused an increase in the frequency of the blocks, and a second dose of the same size abolished both block and sinus arrhythmia (Fig. 9, E, F, G).

Previous administration of strophanthin or digitalis did not appear to produce either qualitative or quantitative changes in the effects produced by atropine.

*Auriculoventricular (P-R) Interval.*—As was expected, this was markedly affected by atropine. By doses which abolished the arrhythmia or blocks and accelerated the sinus rate it was usually but not always shortened, while after doses causing auriculoventricular blocks it was, as a rule, lengthened. There were, however, many exceptions. Apparently the effect of atropine on the P-R interval was often complicated by the effect which acceleration or retardation of the sinus rate normally exerts in retarding or accelerating the rate of conduction (Table II). As may be seen in a number of instances (Experiments 65, 67, 68, and 69, Table II), and as is illustrated in the chart from Experiment 69 (Text-fig. 1), there is often the same lack of parallelism in the effects on sinus rate and conduction time (P-R interval), to which attention has been called by Cohn and Fraser (16). This may be due either to a quantitatively unequal effect on different parts of the vagus center or on the different vagus nerve endings, the former of which are stimulated while the latter are depressed.

TABLE II.  
*Effect of Atropine on Conduction Time, Rhythm, and Sinus Rate in Dogs.*

Dog No.	Strophanthin previously given.	P-R interval before.		Atropine per kilo.	P-R interval after.		Rhythm.		Effect on sinus rate.	Effect on P-R time.	
		sec.	Average. sec.		mg.	sec.	Average. sec.	Before.			After.
62	+	0.11 -0.16	0.092	0.016	0.11 -0.17 0.12 -0.18 0.12 -0.20 0.10 -0.18 0.11 -0.15 0.11 -0.13		S-A and occasional A-V block. "	A-V block.	+	Increased.	
	+	0.11 -0.16		0.033 in 11 min.				Regular.	++	" then diminished.	
	+	0.085-0.10	0.092	0.016	0.08 -0.09 0.08 -0.095 0.08 -0.085 0.11 -0.125 0.08 -0.095 0.12 -0.14 0.10 -0.11 0.13 -0.15 0.12 -0.145 0.11 -0.14 0.09 -0.11 0.14 -0.16	0.084 0.094 0.082 0.115 0.087 0.13 0.108 0.133 0.129 0.122 0.10 0.148	S-A " " " " " " " " " " "	S-A Regular. " " " S-A Regular. " S-A A-V block. Regular. Occasional A-V block. Regular. "	0 + ++ ++ ++ ++ + Slight + + ++ 0 + ++	Diminished. 0 Diminished. 0 Diminished. Increased. Diminished. Increased. " " Diminished. Slightly increased.	
	+	0.085-0.10	0.092	0.033 in 2½ min.							
	+	0.085-0.10	0.092	0.05 " 12 "							
65	+	0.11 -0.125	0.115	0.025	0.08 -0.095 0.12 -0.14 0.10 -0.11 0.13 -0.15 0.12 -0.145 0.11 -0.14 0.09 -0.11 0.14 -0.16	0.087 0.13 0.108 0.133 0.129 0.122 0.10 0.148	" " " " " " " "	S-A Regular. " " " S-A Regular. " S-A A-V block. Regular. Occasional A-V block. Regular. "	0 + ++ ++ ++ ++ + Slight + + ++ 0 + ++	Diminished. 0 Diminished. 0 Diminished. Increased. Diminished. Increased. " " Diminished. Slightly increased.	
	+	0.105-0.12	0.118	0.025							
	0	0.12 -0.14	0.124	0.006							
	0	0.12 -0.14	0.124	0.012							
	+	0.12 -0.13	0.125	0.012 in 3 min.							
67	0	0.11 -0.12	0.114	0.006	0.13 -0.15 0.12 -0.145 0.11 -0.14 0.09 -0.11 0.14 -0.16	0.133 0.129 0.122 0.10 0.148	" " " " "	S-A A-V block. Regular. Occasional A-V block. Regular. "	Slight + + ++ 0 + ++	Increased. " Diminished. Slightly increased.	
	0	0.11 -0.12	0.114	0.012 in 6 min.							
	0	0.11 -0.12	0.114	0.033 " 21 "							
	+	0.13 -0.17	0.143	0.006							
	+	0.13 -0.17	0.143	0.012 in 10 min.							
69	+	0.13 -0.17	0.143	0.02 in 15 "	0.13 -0.17 0.11 -0.115	0.15 0.112	" "	Regular. "	++ ++	Diminished.	
	+	0.13 -0.17	0.143	0.02 " 15 "							



68	0	0.09 -0.10	0.0925	0.007	0.0825-0.16	0.098	S-A	Occasional A-V block. "	+	Slightly increas- ed.
				90 sec. later.	0.07 -0.095	0.086	"	"	+	Slightly de- creased.
	0	0.09 -0.10	0.0925	0.013 in 5 min.	0.07 -0.095	0.083	"	Regular.	++	Diminished.
69	0	0.115-0.15	0.126	0.01 " 2	0.11 -0.13	0.119	"	S-A	0	Slightly dimin- ished.
	0	0.115-0.15	0.126	0.02 " 9	0.15 -0.17	0.157	"	" " and occa- sional A-V block.	+	Increased.
	0	0.115-0.15	0.126	0.05 " 35	0.10 -0.11	0.103	"	Regular.	++	Diminished.
	+	0.10 -0.125	0.108	0.012 " 5	0.105 -0.14	0.115	"	A-V block.	+	Increased.
	+	0.10 -0.125	0.108	0.016 " 7	0.11 -0.20	0.15	"	S-A, no A-V block.	+	"
	+	0.10 -0.125	0.108	0.05 " 42	0.095 -0.10	0.098	"	Regular.	++	Diminished.

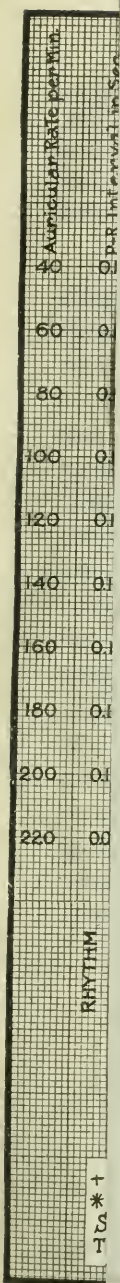


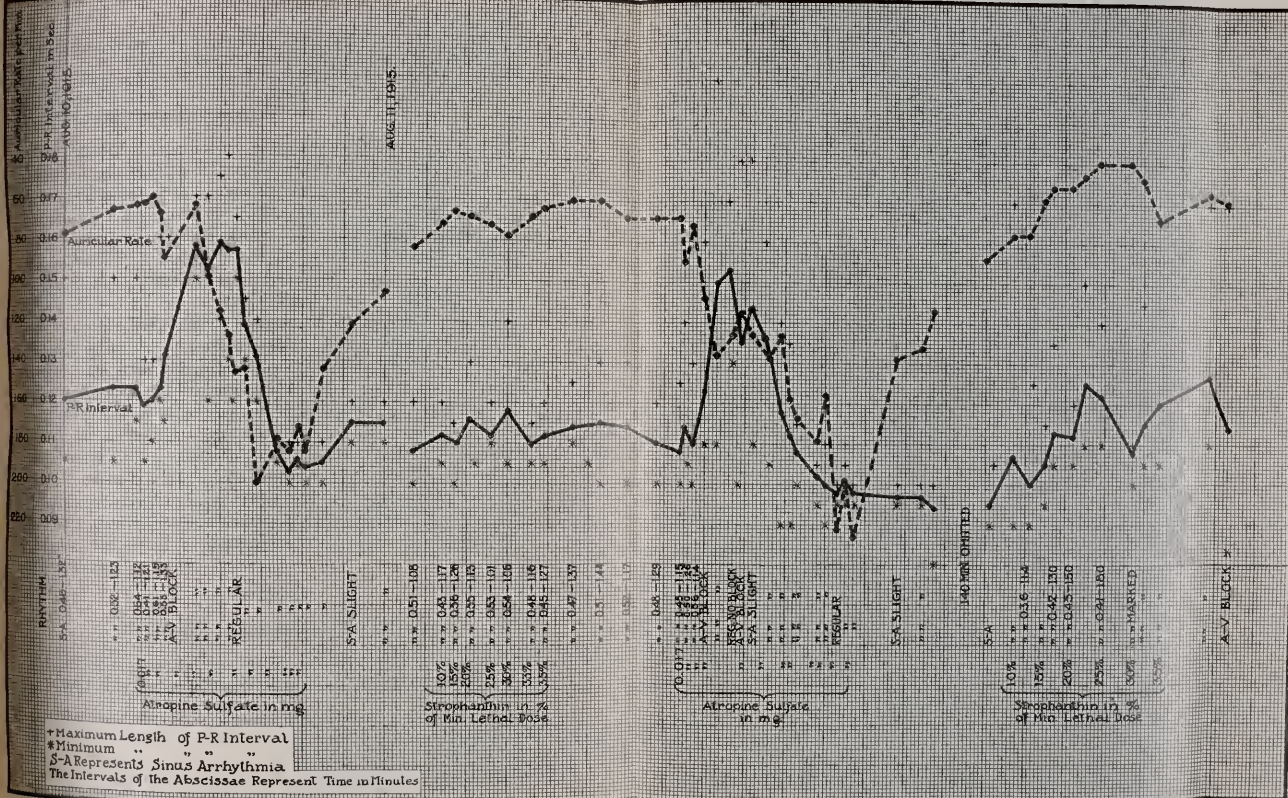
*P Wave.*—In some of the experiments, but not in all, the P wave underwent distinct modifications under the influence of atropine. Usually when atropine caused an auriculoventricular block the P wave became less positive or isoelectric or negative (Figs. 9, F and 10, B). It became positive again when subsequent peripheral vagal depression manifested itself (Figs. 9, G and 10, C). Doses which caused marked acceleration and regular rhythms usually caused the P wave to become more strongly positive.

*Ventricular Complex.*—The ventricular complex was at times altered in various ways. The T wave during the stage of vagus stimulation showed no changes of significance, perhaps because in all such observations the vagus was already acting rather powerfully before atropine was administered. Later, however, when vagus control had been abolished by atropine, the T wave in most instances became decidedly more positive (compare Fig. 6, E with 6, D). When the ventricular complexes were normal before the administration of atropine, no other changes were noted except in two instances.

In Experiment 64, although large amounts of strophanthin (71 per cent of the minimum lethal dose in 4 hours and 29 minutes, and 77 per cent in 5 hours and 14 minutes) had been given, the ventricular complexes were normal. Two injections of atropine, given 48 minutes apart, were each followed by the occurrence of ectopic beats of the right ventricular type, which persisted each time for less than 10 minutes (Fig. 3, D and E). In Experiment 83, 18½ minutes after 39 per cent of the lethal dose of digipuratum had been given and 60 seconds after a second injection of 0.064 mg. of atropine sulfate incomplete heart block appeared (Fig. 6, F). Ectopic ventricular beats appeared 5½ minutes later (Fig. 6, G). A third injection of 0.125 mg. given 7 minutes later caused their disappearance, but they returned 60 seconds later, then disappeared for a time, but returned from time to time for the next hour. During this period the acceleration of the heart action indicated that the peripheral vagus depressing effect of the atropine was still present.

Whether it is correct to attribute the occurrence of these ectopic contractions to the previously administered strophanthin or digipuratum alone, or to a combination of the action of atropine and the other drug cannot be definitely determined. In favor of the latter explanation are the experiences in two experiments. In Experiment 83, although digipuratum had not been given for a considerable period (24 minutes and 3 minutes) each of the two injections of atropine was followed by ectopic beats, and in Experiment 64 atropine appeared twice to cause their occurrence. The explanation appears to be that the removal of vagus control permits the tertiary centers in the ventricle, already excited by







strophanthin or digipuratum, to inaugurate ventricular contraction. Against this view must be considered the fact that in numerous other experiments under apparently similar conditions atropine did not cause the occurrence of ectopic contractions, but, as will be shown below, appeared at times to prevent them, or to cause their disappearance (Danielopolu (17)).<sup>14</sup>

In a number of experiments, when ectopic contractions appeared after the administration of strophanthin or digitalis, atropine caused them to disappear or to become less frequent (Fig. 4, D, E, and F). At times when large doses of strophanthin or digipuratum had caused a constant succession of ectopic beats, the administration of atropine was followed by a change in the shape of the ventricular complex so that it more closely resembled that of a nomotopic contraction (Fig. 5, D and E), but in other instances under apparently similar conditions this change did not occur. In Experiment 71, for instance, although at an earlier stage atropine caused the discontinuance of ectopic beats, it lost this power later. Both before and after atropine failed, this result was accomplished by giving amyl nitrite. In Experiment 73, after large doses of strophanthin had caused ectopic ventricular contractions, atropine produced about the same effects as did amyl nitrite. Early in the experiment both altered the complex somewhat but later produced little or no effect. A comparison of all the observations made with these two drugs demonstrates the fact that amyl nitrite possesses, to a much higher degree than does atropine, the power of inhibiting or altering ectopic ventricular contractions.

#### SUMMARY.

##### *The Effects of Strophanthin and of Digitalis.*

1. In intact dogs the dose which caused slowing and other signs of vagus stimulation without causing ectopic contractions usually

<sup>14</sup>Danielopolu (17) reports a case in which the administration of 1.5 mg. of atropine sulfate regularly relieved a bigeminy produced by digitalis or prevented its development even after doses of digitalis larger than those which regularly caused the appearance of bigeminy. In this instance the heart rate was increased markedly (from 70 to 130) by atropine. When the heart became slow again the bigeminy returned. In the experiments under discussion there was no such increase in the heart rate following the administration of atropine.

lay between 30 and 40 per cent of the minimum lethal dose for Thoms *g*-strophanthin<sup>15</sup> and for digipuratum<sup>16</sup> and a fluid extract of digitalis,<sup>16</sup> given intravenously in dilute solution in about 15 minutes.

2. In dogs strophanthin and digitalis when given intravenously produced typical alterations in the electrocardiogram almost immediately and with equal rapidity.

3. In this series the changes in the electrocardiogram following the administration of 25 to 40 per cent of the minimum lethal dose of strophanthin or digitalis often passed off within an hour or two and with few exceptions were not visible in electrocardiograms taken 20 hours later. The effects of digitalis did not appear to be more lasting than those of strophanthin. As there were marked differences in the persistence of the action of each of these drugs within its own series, this conclusion is provisional.

4. A persistent auriculoventricular block (without the occurrence of ectopic beats) was obtained only exceptionally, but the administration of 30 to 40 per cent of the minimum lethal dose of either of these drugs was usually followed by the occurrence either singly or together of occasional auriculoventricular blocks or exaggerated sinus arrhythmia or sinus block, and by pronounced slowing of the sinus rate.

5. Occasionally, with or without previous slowing or the occurrence of ectopic beats, these doses caused a marked acceleration of the sinus rate.

6. The auriculoventricular (P-R) time was usually lengthened but may be shortened.

7. The P wave may become less positive, isoelectric, or negative.

8. The T wave most often became negative before or during the stage of slowing but frequently became or remained positive during this stage. When the sinus rate increased or when occasional ectopic beats occurred, this wave almost always became decidedly more positive. At times it varied rapidly and decidedly within a period of a few seconds.

<sup>15</sup> 0.12 mg. per kilo.

<sup>16</sup> Twelve frog units per kilo.

9. Ectopic ventricular beats occurred in a number of instances after doses ranging from 19 to 40 per cent of the minimum lethal dose, and usually but not always when more than 40 per cent had been injected within 15 to 30 minutes.

10. Previous administration of either of these drugs did not appear to cause either a quantitative or qualitative alteration in the reaction of the heart to amyl nitrite or atropine (with the exception that such previous administration appeared responsible for the fact that in one experiment the inhalation of amyl nitrite, and in two others the injection of atropine, was followed by the occurrence of ectopic beats).

### *The Effects of Amyl Nitrite.*

1. In intact dogs, both before and after the administration of strophanthin or of digitalis, the inhalation of amyl nitrite usually caused at first (probably largely reflexly) a pronounced increase of vagus tone shown by slowing of the sinus rate, exaggerated sinus arrhythmia, or auriculoventricular block. Later the sinus rate was accelerated and arrhythmia or blocks disappeared (probably due to vasodilatation and diminished vagus tone).

2. The auriculoventricular (P-R) time was usually lengthened at first and later was shortened.

3. The P wave often at first became shorter, isoelectric, or negative, but later became much more positive.

4. The T wave at first often became less positive or more negative, but later became strongly positive.

5. When the previous administration of not too large doses of strophanthin or digitalis caused the occurrence of ectopic ventricular beats, the inhalation of amyl nitrite usually caused their temporary disappearance. Even when large doses of these drugs caused a constant succession of ectopic contractions of various types, its inhalation was often followed by a temporary return of nomotopic cycles or by a change in the form of the ventricular complexes so that they more closely resembled that of nomotopic ones.

6. Only large amounts of amyl nitrite appear to have the power to prevent ectopic ventricular beats or to alter their type.



*The Effects of Atropine.*

1. In intact dogs before and after the administration of strophanthin or digitalis, small doses of atropine usually caused auriculo-ventricular block (or exaggerated it if already present). Larger doses, after first producing the same effect as the small ones, caused an increased sinus rate and abolished blocks or sinus arrhythmias if present.

2. The P-R time was usually lengthened by small doses and shortened by larger ones.

3. The P wave sometimes became shorter at first (less positive), isoelectric, or negative, but later (when the peripheral vagus paralysis ensued) it became positive again, often much more so than it was originally.

4. When ectopic contractions were present as a result of previous administration of strophanthin or digitalis, atropine, like amyl nitrite, often caused their disappearance, or altered the form of the ventricular complex, but was less efficient in this direction than amyl nitrite.

5. Occasionally the injection of atropine into dogs, which had previously received strophanthin or digitalis, appeared to facilitate or bring on the occurrence of ectopic ventricular contractions.

I wish to acknowledge my indebtedness to Dr. Alfred E. Cohn for his courtesy and assistance during the experiments and in the preparation of the manuscript.

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#### EXPLANATION OF PLATES.

In all the figures (except Fig. 9, A to G) divisions of the abscissæ equal 0.04 second. In Fig. 9, A to G the time is indicated by the lowest line, each interval representing 0.2 second. Divisions of the ordinates equal  $10^{-4}$  millivolts. In Fig. 1, B to G the upper curve represents the blood pressure as recorded by a Harvard membrane manometer, a rise in the curve denoting a fall in the blood pressure. All the curves were made with Lead II (right fore leg to left hind leg).

#### PLATE 59.

Fig. 1, A to G (Experiment 85). In the upper curve, which is that of the blood pressure, a rise of the curve indicates a fall in the blood pressure.

A and B are controls. In A there is a spontaneous auriculoventricular block.

C was taken when amyl nitrite had been inhaled for 90 seconds. The blood pressure has fallen and as a consequence the heart beats more rapidly than in the control until this increase in heart rate leads to a rise in blood pressure which is followed by an auriculoventricular block. The P waves have become much taller than in the control curves.

D was taken 60 seconds after C when the inhalation had lasted  $2\frac{1}{2}$  minutes. The blood pressure has fallen in spite of the increased heart rate, and blocks and sinus arrhythmia have been abolished.

E was taken 1 minute after 29 per cent of the minimum lethal dose of a fluid extract of digitalis had been injected in 72 minutes. It shows an auriculoventricular block and a sinus arrhythmia.

F was taken 4 minutes after E when amyl nitrite had been administered for  $3\frac{1}{4}$  minutes. The sinus rate has increased and the blood pressure has fallen decidedly, but the curve shows one auriculoventricular block and some sinus arrhythmia.

G was taken 30 seconds after F when the inhalation had lasted  $3\frac{3}{4}$  minutes. The blood pressure has fallen still further, and arrhythmia and blocks are abolished. The sinus rate is markedly accelerated, the P-R interval is shorter, the P waves are much taller, and the T waves are more positive than in E.

## PLATE 60.

FIG. 2, A and B (Experiment 56).

A is the control.

B shows auriculoventricular block with ectopic ventricular beats developing 6 minutes after the injection of 40 per cent of the minimum lethal dose of strophanthin. It also shows a negative T wave and the P-R interval increased to 0.17 second.

FIG. 3, A to E (Experiment 64).

A is the control. B and C serve also as controls to D and E.

B was taken when 71 per cent of the minimum lethal dose of strophanthin had been administered in 4 hours and 29 minutes. It shows slowing of the sinus rate and marked sinus arrhythmia with negative T waves.

C was taken 4 minutes and 20 seconds after B, and 80 seconds after the injection of 0.19 mg. of atropine sulfate. It shows abolition of sinus arrhythmia and marked acceleration of sinus rate, increase in height of the P waves, and change in form of the T waves. The P-R interval is almost unaltered.

D, taken 2 minutes and 50 seconds after C, shows ectopic ventricular beats.

E, taken 3 minutes after D, shows ectopic ventricular beats.

## PLATE 61.

FIG. 4, A to F (Experiment 71).

A is the control.

B was taken after 44 per cent of the minimum lethal dose of strophanthin had been injected in 23 minutes. It shows ectopic ventricular beats.

C was taken 80 seconds after B. Amyl nitrite has been inhaled for 40 seconds. It shows the disappearance of ectopic beats.

D was taken 8 minutes after C. It shows a return of the rhythm seen in B. No additional strophanthin has been given.

E was taken 16 minutes after D; 5½ minutes after 50 per cent strophanthin had been injected in 43 minutes; 15 minutes after a first injection of atropine sulfate, 0.0125 per kilo; and 2 minutes after a second injection of the same size. It shows the altered form of the ventricular complex.

F was taken 3 minutes after E and 30 seconds after the injection of a third dose of atropine sulfate, 0.025 mg. per kilo (0.05 mg. per kilo in all). It shows normal cycles.

FIG. 5, A to E (Experiment 73).

A is the control.

B was taken 6 minutes after the injection of 96 per cent of the minimum lethal dose of strophanthin in 1 hour and 47 minutes. It shows ectopic ventricular beats of different types.

C was taken 90 seconds after B, when amyl nitrite had been inhaled for 60 seconds. It shows alteration in the form of the ventricular complexes.

D was taken 90 seconds after C. No amyl nitrite was given for 90 seconds. It shows the return to curves similar to those in B before inhalation of amyl nitrite.

E was taken 3 minutes after D, 30 seconds after the injection of atropine sulfate, 0.07 mg. per kilo. It shows ventricular complexes like those seen in C, following the inhalation of amyl nitrite.

#### PLATE 62.

FIG. 6, A to G (Experiment 83).

A is the control.

B was taken 4 minutes after 37 per cent of the minimum lethal dose of digipuratum had been injected in 2 hours and 25 minutes. It shows slowing of the rate with sinus arrhythmia.

C was taken 2 minutes after B. Amyl nitrite had been inhaled for 60 seconds. The curve shows abolition of sinus arrhythmia, acceleration of sinus rate, increase in height of P waves, and strongly positive T waves.

D was taken 19 minutes after C, 15 minutes after 39 per cent of the minimum lethal dose of digipuratum had been injected during 2 hours and 46 minutes.

E was taken 90 seconds after D and 30 seconds after the injection of atropine sulfate, 0.012 mg. per kilo. It shows slight variation in the height of the P waves and a marked change in the T waves which are much more positive than in D.

F was taken 2 minutes after E, 60 seconds after a second dose of atropine sulfate, 0.012 mg. per kilo. It shows three auriculoventricular blocks and further change in the T waves.

G was taken 5½ minutes after F. It shows ectopic ventricular contractions, alternating with cycles like those in F.

#### PLATE 63.

FIG. 7, A to D (Experiment 83).

A is the control.

B was taken 10 minutes after the injection of 20 per cent of the minimum lethal dose of digipuratum in 34 minutes. It shows ectopic beat and an auriculoventricular block, also tall T waves. The P-R interval is increased.

C was taken 4 minutes after B when amyl nitrite had been inhaled for 20 seconds. It shows an ectopic beat and an auriculoventricular block. The T wave has become less positive.

D was taken 20 seconds after C when amyl nitrite had been inhaled for 40 seconds. The sinus rate accelerated, the P-R interval shortened, and the T wave became strongly positive. The P waves are now all very tall.

FIG. 8, A, B, and C (Experiment 53).

A is the control.

B was taken 70 seconds after the commencement of inhalation of amyl nitrite. It shows an R-R interval of 4.02 seconds.

C was taken at the end of the inhalation, lasting 7 minutes and 40 seconds. It shows a greatly increased rate and regular rhythm. The P-R interval shortened to 0.08 second.

## PLATE 64.

FIG. 9, A to G (Experiment 62).

A is the control.

B was taken immediately after the injection of 19 per cent of the minimum lethal dose of strophanthin. It shows an auriculoventricular block, an ectopic ventricular beat, a change to a negative T wave, and a slightly lengthened P-R interval.

C was taken 2 hours and 10 minutes after B. 36 per cent of the minimum lethal dose had been injected in 3 hours and 15 minutes. It shows exaggerated sinus arrhythmia, P waves varying from strongly positive to isoelectric, and negative T waves.

D was taken 4 minutes later; amyl nitrite had been inhaled for 30 seconds. The arrhythmia is abolished, the rate markedly increased, the P-R time shortened to 0.08 second, all P waves are strongly positive, and T waves changed from negative to diphasic.

E was taken 23 minutes and 20 seconds after D. 40 per cent of the minimum lethal dose of strophanthin has been injected in 3 hours and 42 minutes. It shows marked sinus arrhythmia, one blocked P wave, P waves varying from strongly positive to isoelectric, and T waves of variable degrees of inversion.

F was taken  $3\frac{1}{2}$  minutes after E and 2 minutes after the injection of 0.016 mg. of atropine sulfate per kilo. It shows much more frequent auriculoventricular blocks, P waves of varying heights, and a lessening in the negativity of the T waves.

G was taken  $12\frac{1}{2}$  minutes after F and 5 minutes after the second injection of 0.016 mg. of atropine sulfate per kilo. It shows marked acceleration of sinus rate, abolition of block, and almost complete merging of T and P waves, both of which are strongly positive.

## PLATE 65.

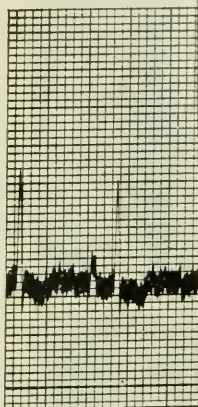
FIG. 10, A, B, and C (Experiment 68).

A is the control.

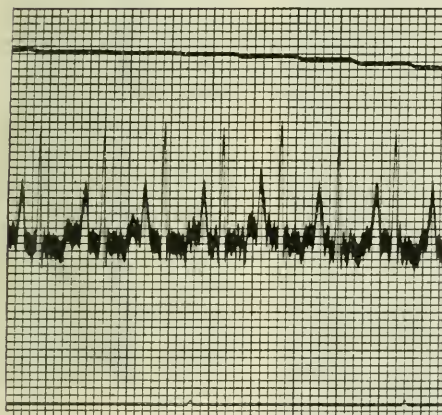
B was taken 30 seconds after the injection of atropine sulfate, 0.006 mg. per kilo. It shows an auriculoventricular block with negative P waves. It also shows P waves changing from negative to positive and back again to negative.

C was taken 7 minutes after B, and 35 seconds after the second injection of atropine sulfate, 0.006 mg. per kilo. It shows accelerated sinus rate, abolition of block and arrhythmia, shortened P-R interval, the P waves strongly positive, and change of the T wave to strongly positive.

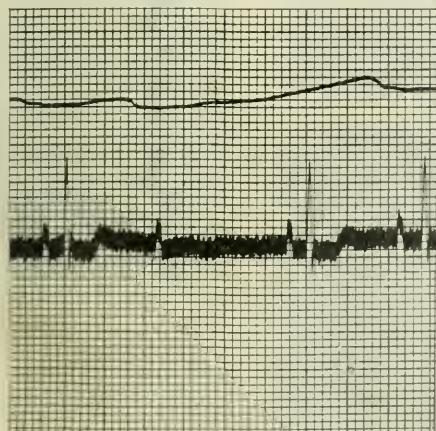




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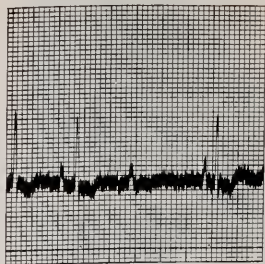


1 C

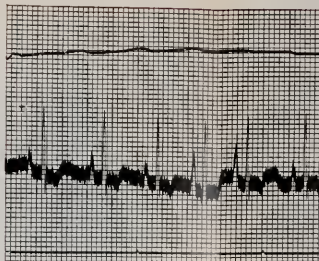


1 E

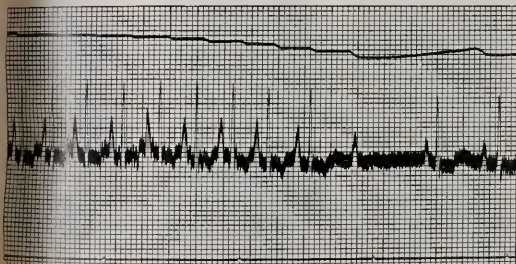




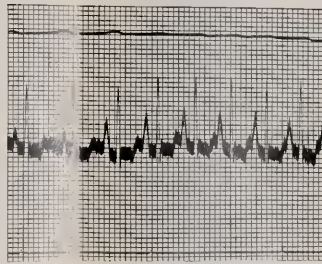
1 A



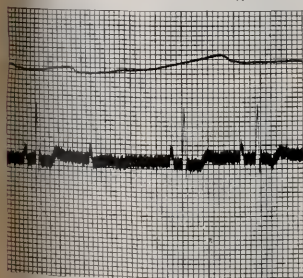
1 B



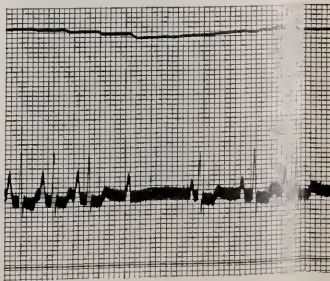
1 C



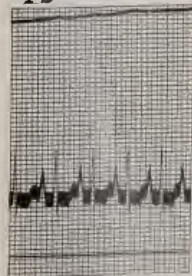
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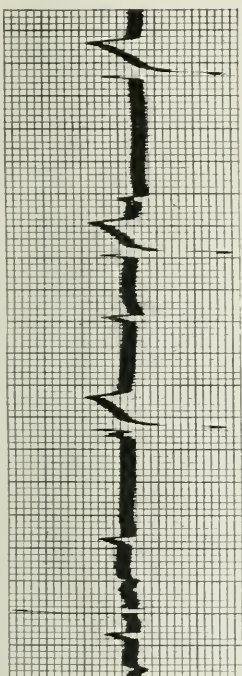


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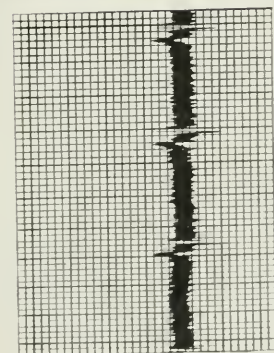


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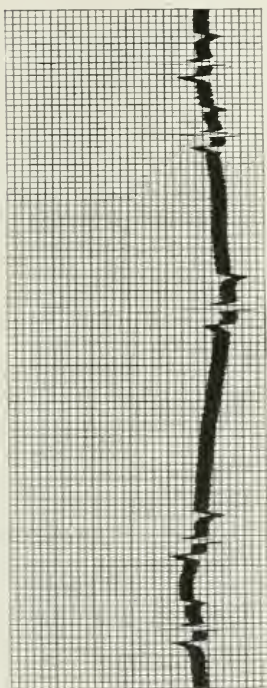
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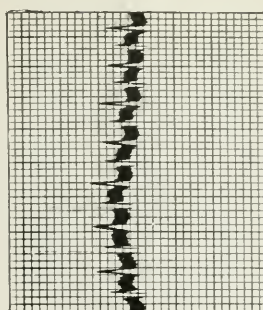
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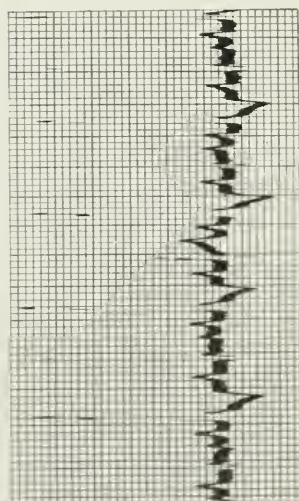
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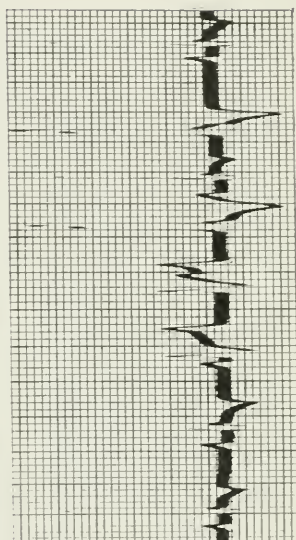
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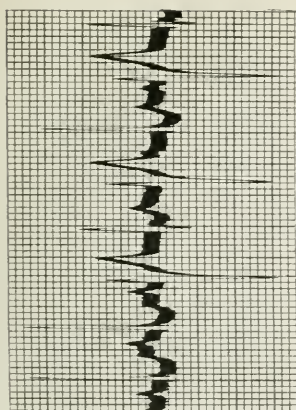


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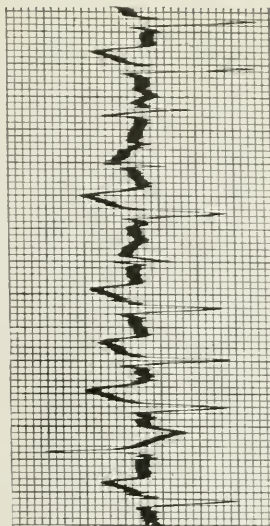
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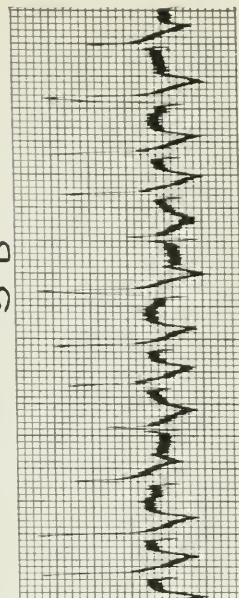




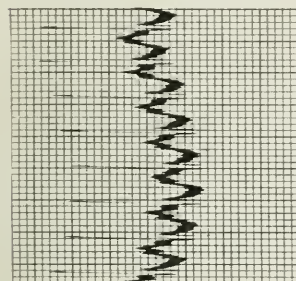
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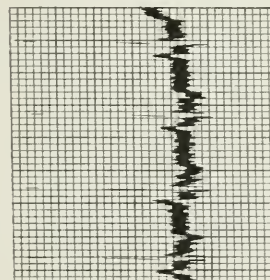
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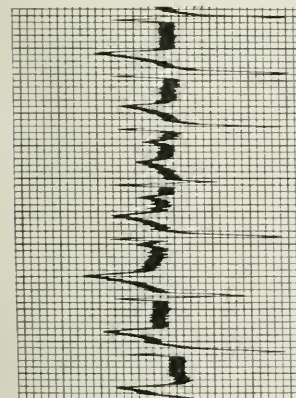
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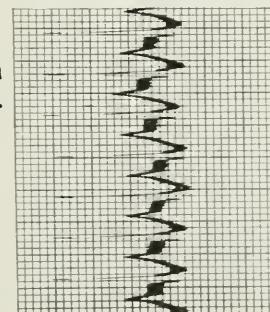
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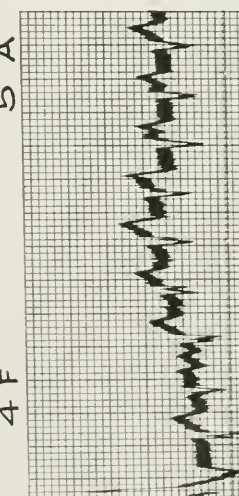
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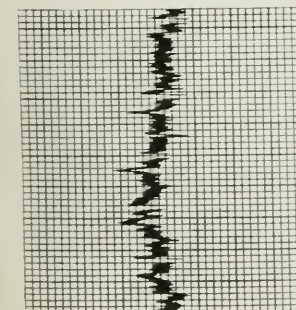
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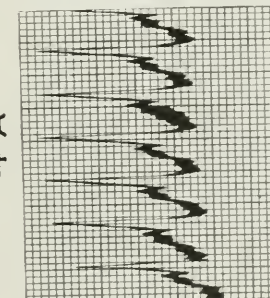
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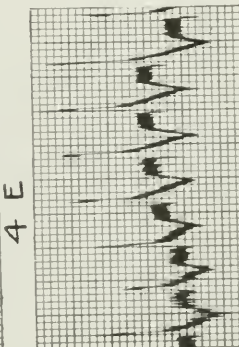
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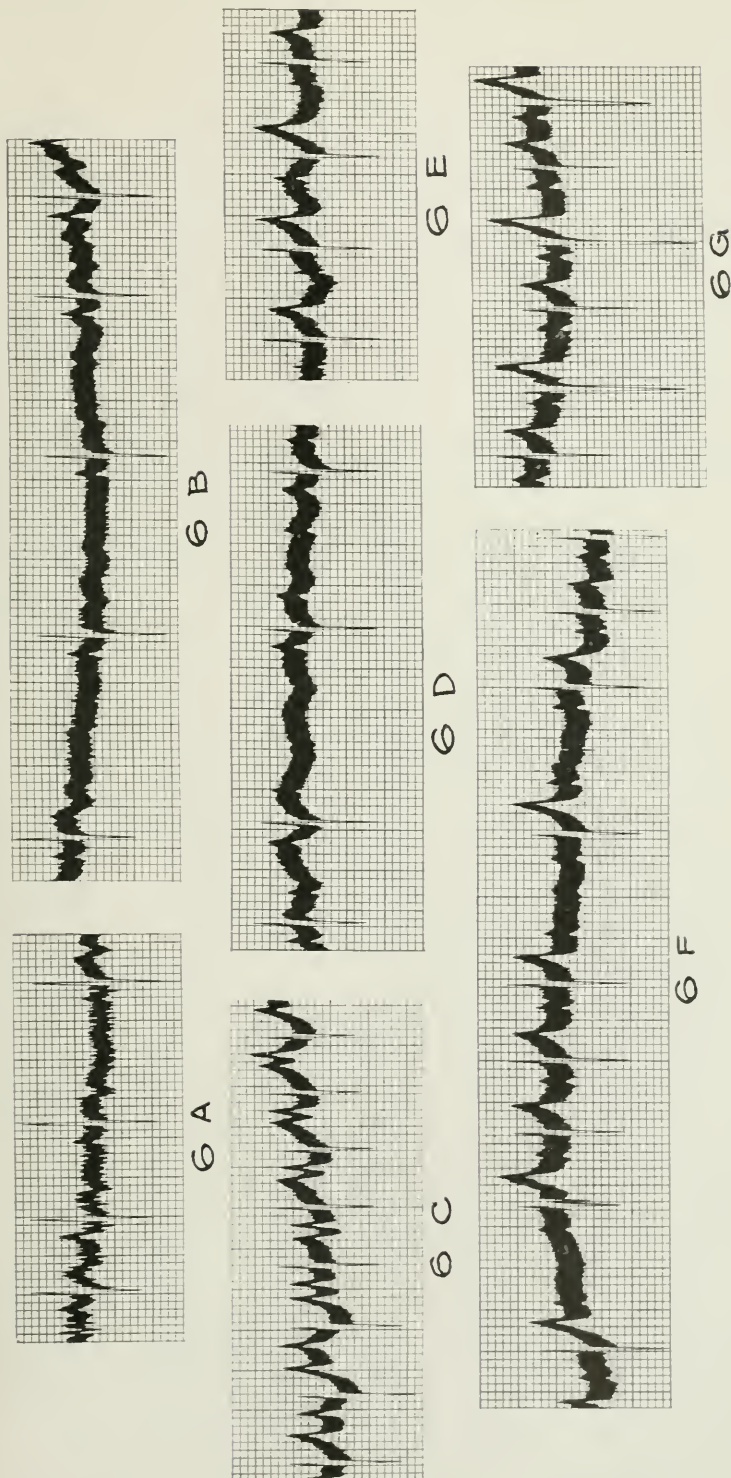
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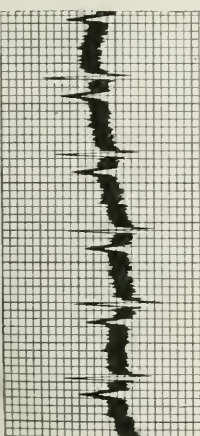


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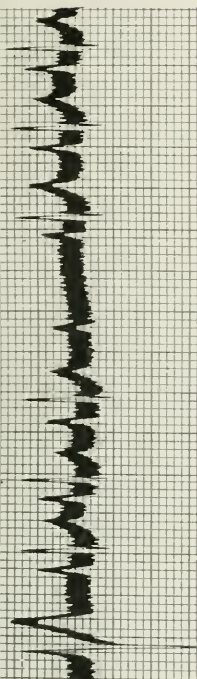




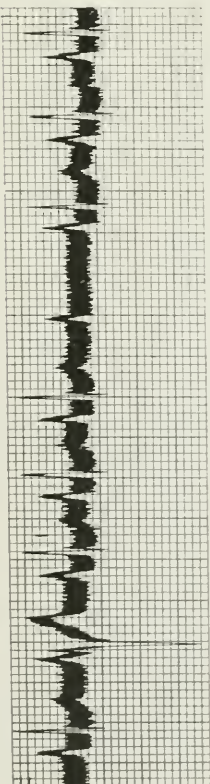




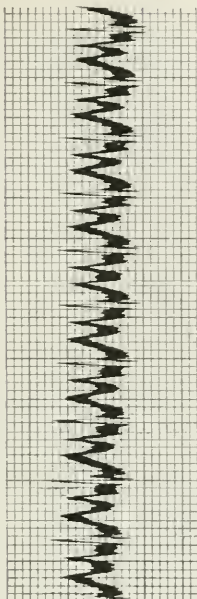
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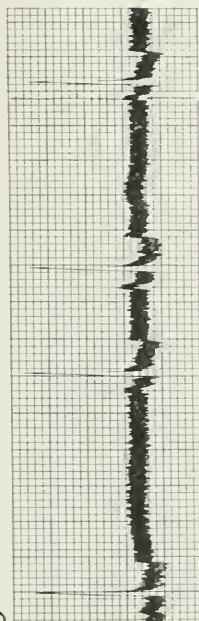
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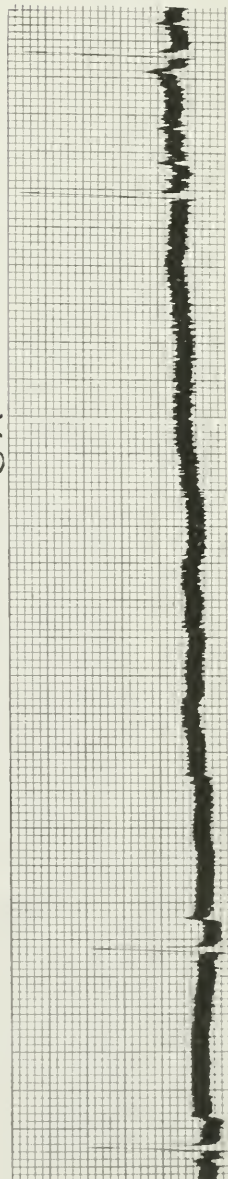
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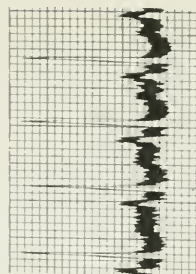
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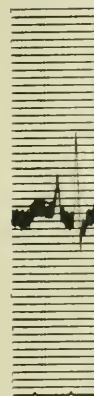


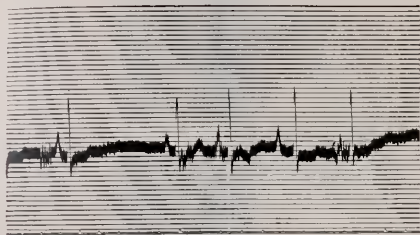
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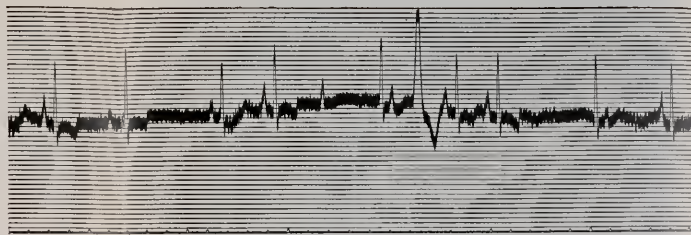
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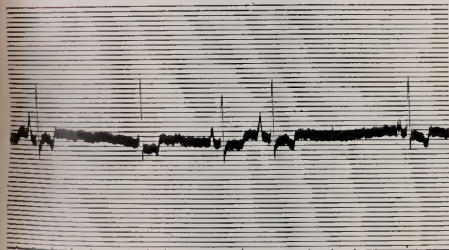




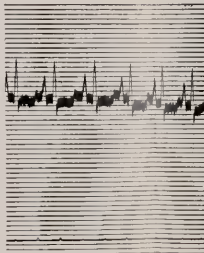
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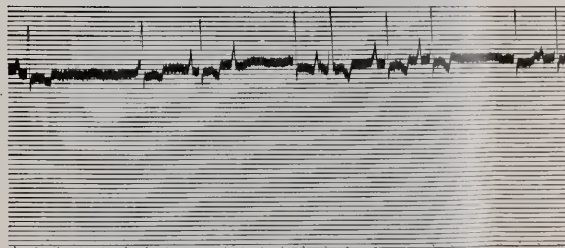
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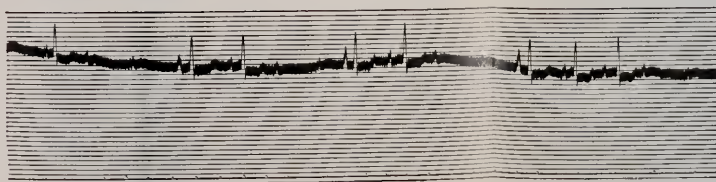
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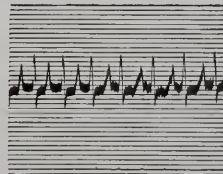
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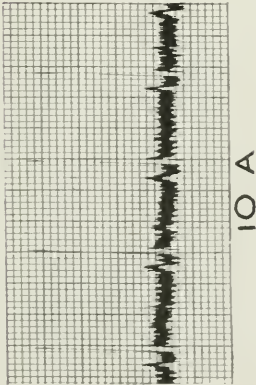
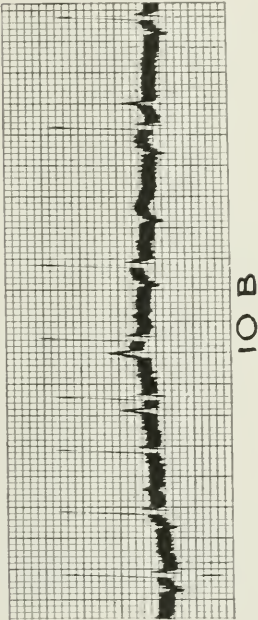
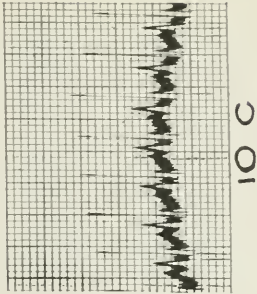
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# SPIROCHÆTA ICTEROHÆMORRHAGIÆ IN AMERICAN WILD RATS AND ITS RELATION TO THE JAPANESE AND EUROPEAN STRAINS.

## FIRST PAPER.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Infectious jaundice has been known for a long time to occur among troops in barracks, among sewer workers, miners, and rice planters, and its entity has been recognized (Larrey, Ozanam, 1849, Monneret, 1859, Laverau, 1865, Lancereau, 1882, Landouzy, 1882, Mathieu, 1886, and Weil, 1886). Weil described four cases with typical symptoms, and the disease is very often called by his name. Weil's disease is characterized by sudden onset of malaise, often intense muscular pains, high fever for several days, followed by jaundice and the appearance of bile pigments, albumin, and casts in the urine; in severe cases epistaxis, subcutaneous hemorrhages, and lymphadenitis are observed.

A disease supposed to be identical with the Weil's disease of Europe is prevalent in Japan, and in 1914 Inada and Ido succeeded in transmitting to guinea pigs the typical experimental disease, accompanied by jaundice, hemorrhages, albuminuria, etc., by inoculating them with the blood of patients suffering from the Japanese form of infectious jaundice. In the blood and various organs of the animals they discovered a new spirochete, which they also found in the human specimens. *Spirochata icterohæmorrhagiæ*, as it was designated by Inada and his associates,<sup>1</sup> has since been proved beyond doubt to be the causative agent of the disease in question. A year and a half later, Uhlenhuth and Fromme,<sup>2</sup> and also Hübener and Reiter,<sup>3</sup> showed that the Weil's disease prevalent among the German soldiers during the present war could also be transmitted to guinea pigs by injecting them with the blood of patients. It was not until 1916, however, that Hübener and Reiter<sup>4</sup> announced the finding of a spirochete in the experimental animal material (liver, blood, kidney, etc.), which they called *Spirochata*

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<sup>1</sup> Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377.

<sup>2</sup> Uhlenhuth and Fromme, *Med. Klin.*, 1915, xi, 1202, 1264, 1296, 1375.

<sup>3</sup> Hübener and Reiter, *Deutsch. med. Woch.*, 1915, xli, 1275.

<sup>4</sup> Hübener and Reiter, *Deutsch. med. Woch.*, 1916, xlii, 1.

*nodosa*. Following the publication in America<sup>1</sup> of the article by Inada and his collaborators, Stokes and Ryle<sup>5</sup> succeeded in transmitting the disease to guinea pigs by inoculating them with the blood of British soldiers in Flanders who had Weil's disease, confirming at the same time the presence of a spirochete closely resembling the *Spirochæta icterohæmorrhagiæ* of the Japanese workers. Stokes, Ryle, and Tytler<sup>6</sup> left the question of the identity of the two strains (the Belgian and the Japanese) open for future discussion. In the meanwhile, Martin and Pettit<sup>7, 8</sup> Costa and Troisier,<sup>9, 10</sup> Garnier and Reilly,<sup>11</sup> Renaux,<sup>12</sup> Merklen and Lioust,<sup>13</sup> Ameuille,<sup>14</sup> Salomon and Neveu,<sup>15</sup> and others, working among the French soldiers on the western front, reported similar clinical and experimental findings. They also considered the spirochete isolated from the French specimens to be closely related to the Japanese strain. On the Italian front numerous cases of jaundice have been observed, and Monti<sup>16</sup> has demonstrated the spirochete in the experimental material. According to MacKenzie, there were at one time a considerable number of cases of infectious jaundice among the Canadian soldiers stationed at Salonica.<sup>17</sup> It is of interest to note that while the mortality among the Japanese is as high as 38 per cent, that of the European soldiers did not exceed 2 to 3 per cent. It seems reasonable to assume that the Japanese strain has already acquired a marked increase in virulence for human subjects, owing probably to a more frequent passage from man to man. Such transmission is more frequent in Japan, for example, among the rice planters and miners with imperfectly protected feet, than in Europe, where exposure to the infection was brought about only through the unsanitary conditions of war.

The entrance of this spirochete into the human body seems to be of comparatively recent occurrence. The discovery of the spirochete in apparently healthy wild rats caught near the infected districts

<sup>5</sup> Stokes, A., and Ryle, J. A., *J. Roy. Army Med. Corps*, 1916, xxvii, 286.

<sup>6</sup> Stokes, A., Ryle, J. A., and Tytler, W. H., *Lancet*, 1917, i, 142.

<sup>7</sup> Martin, L., and Pettit, A., *Presse méd.*, 1916, 569.

<sup>8</sup> Martin and Pettit, *Bull. Acad. méd.*, 1916, lxxvi, 247.

<sup>9</sup> Costa, S., and Troisier, J., *Compt. rend. Soc. biol.*, 1916, lxxix, 1038.

<sup>10</sup> Costa and Troisier, *Bull. et mém. Hôp. Paris*, 1916-17, xl, series 3, 1928.

<sup>11</sup> Garnier, M., and Reilly, J., *Bull. et mém. Hôp. Paris*, 1916-17, xl, series 3, 2249.

<sup>12</sup> Renaux, E., *Compt. rend. Soc. biol.*, 1916, lxxix, 947.

<sup>13</sup> Merklen, P., and Lioust, C., *Bull. et mém. Hôp. Paris*, 1916-17, xl, series 3, 1865.

<sup>14</sup> Ameuille, P., *Bull. et mém. Hôp. Paris*, 1916-17, xl, series 3, 2281.

<sup>15</sup> Salomon, M., and Neveu, R., *Compt. rend. Soc. biol.*, 1917, lxxx, 272.

<sup>16</sup> Monti, A., *Boll. Soc. med. chir. Pavia*, 1916, Nos. 3-4.

<sup>17</sup> Personal communication.

seems to support the theory that the disease was originally epizootic among certain rodents, particularly wild rats, and after a long sojourn in this species of hosts its virulence for these animals has been reduced to such an extent as to cause the latter no inconvenience, or, at least, a state of tolerance for the spirochete has developed.

Inada, Ido, Hoki, Ito, and Wani<sup>18</sup> found nearly 30 per cent of all the rats examined to be infected; that is, to carry the spirochete in their kidneys. Stokes Ryle, and Tytler,<sup>6</sup> as well as Martin and Pettit,<sup>19</sup> were able to demonstrate the spirochete in rats captured on the western front, not only in those taken in the immediate neighborhood of the infected zone, but also in rats captured in localities some distance from the trenches.

In America, especially the United States, there have been few epidemic or endemic cases of infectious jaundice reported from various quarters of the continent (Toronto, Middle Western and Southern United States) and from Cuba, but it was not known whether or not these cases corresponded with those found in Europe and Asia. The discovery of the specific pathogenic agent now enables us to answer this question experimentally.

#### *Isolation of the Organism.*

We have collected a large number of wild rats in this country and removed their kidneys for the purpose of ascertaining whether or not the organs contained the spirochete which causes the typical experimental lesions characteristic of the organism of infectious jaundice. Leaving the experimental details for a future communication, it may be well to state here briefly that by inoculating the emulsion made of the kidneys of forty-one wild rats into fifty-eight guinea pigs during the last 3 months, we have been able to produce in three groups of guinea pigs (four in each group) a typical icterohemorrhagic spirochetosis altogether identical with the findings in the guinea pigs which died of the injections of the Japanese and Belgian strains of *Spirochæta icterohæmorrhagiæ*.

Since it was our practice to make an emulsion from the kidneys (eight) of four wild rats and inject the same into four to six normal

<sup>18</sup> Inada, R., Ido, Y., Hoki, R., Ito, H., and Wani, H., *J. Exp. Med.*, 1916, xxiv, 485.

<sup>19</sup> Martin and Pettit, *Compt. rend. Soc. biol.*, 1917, lxxx, 10.

guinea pigs, it is difficult to say whether the icterohemorrhagic spirochetosis produced in some of the guinea pigs in each group was due to one or more of the rats employed. It would have been better if we had inoculated several guinea pigs with the emulsion of kidneys from each rat, but this would have involved a large number of guinea pigs, and hence our method of mixing four in each group. Since the successful isolation of the organisms from three of the pooled groups, we have resorted to individual tests, the results of which will be reported later.

The strain of spirochete isolated from the American wild rats caught in the vicinity of New York City produced death in guinea pigs within 9 to 12 days, attended by marked jaundice, cholemia, choluria, and extensive hemorrhages in various viscera. The temperature seldom exceeds 39°C.; that is, it does not show the fever which usually precedes the collapse and the appearance of jaundice in the guinea pigs inoculated with the Japanese or Belgian strains, which have at this time been passed through many guinea pigs. The spirochetes are found to be abundant in the internal organs, but are only moderately numerous in the blood. In the urine of some guinea pigs, which succumbed to the experimental icterohemorrhagic spirochetosis, varying numbers of the organisms were found in the urinary casts.

The morphology of the organism corresponds with that of the Japanese and Belgian strains, with which we were able to compare it.<sup>20</sup> Its elementary structure is that of a closely wound slender cylindrical thread with gradually tapering ends, averaging 9 by 0.25  $\mu$ . Individuals of 3 to 4  $\mu$  or 20, 30, and even 40  $\mu$  are met with in a culture. The number of coils is greater in a given length than that of any spirochete hitherto known. It is so closely wound that within 5  $\mu$  there are 10 to 12 coils. Near the extremities, the coils become closer. They are never very deep, and in general, the aspect of the whole body is that of a transversely barred chain of streptococci. The winding is rarely seen distinctly, although it can be brought out well by a carefully fixed stained preparation (osmic vapor fixation

<sup>20</sup> I am greatly indebted to Dr. Victor G. Heiser for the Japanese strain and to Dr. Alexis Carrel for the Belgian strain. The Japanese strain was kindly furnished by Dr. M. Miyajima, and the Belgian by Dr. Carl Browning, who obtained it from Dr. Adrian Stokes.



and Giemsa stain), or under powerful dark-field illumination. It should be noted that the description of the organism by most authors leaves this point unclear, and so far no satisfactory microphotograph has been reproduced. The movement and customary position of the organism in a free space are characteristic. Active specimens show a straight body with one or both ends curved in the form of a semi-circle. The length of the hook at the end varies somewhat but is usually about 3 to 5  $\mu$ . While in motion, the organism, without relaxing its elementary minute windings, rotates around its axis, making about two to four turns per second, giving the impression of a drawn out figure eight. The movement is bipolar, and its direction alternates at short intervals. When passing through a semisolid medium, such as fibrin or soft agar, the body of the spirochete assumes a wavy spiral not unlike the *refringens* type. The number of waves may vary from a few broad ones to as many as five or six in some of the long individuals found in a culture. The movements are brusque and erratic, changing suddenly the direction of progression. At times, the organism moves about with one end taking various directions. The facility with which these spirochetes travel through the fibrin or semisolid agar medium is without a parallel among any known spirochetes. The body is absolutely flexible. There is a distinct halo around the organism, but no membrane has so far been demonstrated. The part of the body which forms the hook terminates in a fine point, but no minute flagellum-like projection could be demonstrated by staining (Loeffler, Pitfield, Casares-Gil, Fontana, etc.), or by dark-field illumination. It is devoid of a terminal filament such as is characteristic of a spironema or treponema, and is resistant to saponin (10 per cent), unlike all other spirochetes. It calls for a new genus, and on account of its fine and minute windings, the name *Leptospira* is suggested.

The hooked ends form one of the most characteristic poses of the organism while rotating on its axis in a free space, but as soon as it meets a solid or semisolid obstacle, it begins to penetrate into it. Its habitat seems to be a porous gelatinous mass of substance, the organisms swarming in and out of it. In a culture the majority of the organisms will be found in a semisolid piece of medium. After death it may retain its position at the moment of death; hooked or



contorted forms, resembling the letters c, s, l, and b, are most frequently met with among immobile or dead organisms. When destroyed by a strong acid or alkali, they lose their minute curves, swell up, and become indistinct and straight. The organism has the power of almost perpetual motion. A culture as old as 3 months still displays as much vigor as does a young one. None of the other varieties of spirochetes have this property in cultures. The organism passes the Berkefeld candle V.

### *Cultures.*

The Japanese investigators<sup>1</sup> succeeded in obtaining a culture of their strains by using a medium similar to that previously introduced by the writer for the spirochete of relapsing fever.<sup>21</sup> Ito and Matsuzaki approved this technique and recommended also the use of blood gelatin or blood agar in various concentrations.<sup>22</sup> In Europe, Reiter<sup>23</sup> cultivated the German strain in the sera of various animals, sometimes diluted several times with isotonic salt solution. Recently, Martin, Pettit, and Vaudremer<sup>24</sup> obtained a culture of the French strain by the use of animal sera, particularly that of beef, diluted with Locke's solution. In all these instances, the surface of the culture medium was covered with a layer of sterile paraffin oil, as recommended by the writer for the cultivation of spirochetes in general. The Belgian strain of Stokes has not yet been cultivated, and Stokes, Ryle, and Tytler particularly laid stress on its resistance to artificial cultivation as a characteristic of the strain.

The writer has been interested in determining the cultural conditions which will give uniform results, and has found that the following methods give the most successful growth, not only with the Japanese, but also with the Belgian (Stokes) and American strains, which were obtained in permanent cultures for the first time by these means.

In our experience, it seemed best to make a distinction between the initial culture or the first generation and the subcultures, since there is a great difference in the readiness with which the first and subsequent generations of the spirochetes grow in culture. For example, all three strains, in our hands, failed to grow, or grew poorly

<sup>21</sup> Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.

<sup>22</sup> Ito, T., and Matsuzaki, H., *J. Exp. Med.*, 1916, xxiii, 557.

<sup>23</sup> Reiter, *Deutsch. med. Woch.*, 1916, xlii, 1282.

<sup>24</sup> Martin, L., Pettit, A., and Vaudremer, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 197.

in the various media recommended by different authors. The best procedure, and one which has always been reliable for securing initial growth was to produce strands of loose fibrin in the fluid culture media by using a small quantity of citrate plasma in combination with the diluted or undiluted serum of a suitable animal. The beneficial effect of a loose fibrin upon the culture of the spirochetes of relapsing fever has already been mentioned by the writer.<sup>21</sup> The dilution of the serum may be made in any proportion above 1:10 by adding a sterile saline (0.9 per cent) Ringer solution, or even plain water. For obtaining the spirochetal material for inoculation, the citrate blood derived from the heart of a guinea pig having the disease is best, although an emulsion of the liver or kidney may also be used. When no secondary bacteria are present, a positive culture can be secured at the first attempt. By using graduated quantities of the infected guinea pig's blood (citrate), the writer secured a good growth in as high a dilution as 1:100,000.

Undoubtedly blood cultures for diagnostic purposes in human cases are feasible with a suitable medium. For this purpose I should recommend two different media, one being apparently as good as the other: (a) rabbit serum 1 part + Ringer or 0.9 per cent sodium chloride solution 3 parts + citrate rabbit plasma 0.5 part, covered with a thin layer of sterile paraffin oil; (b) the same, except for the use of 0.5 to 1.0 part of neutral or slightly alkaline agar (2 per cent), which should be added while in a liquid state and quite hot (60–65°C.) in order to get a uniform mixture of the agar. These culture media, because of the paraffin oil layer, can be preserved at room temperature for many months, though a cooler place is better. They may be inoculated with suspected blood by introducing a quantity which is regarded as adequate in each instance. In the case of an infected guinea pig, the detection of the spirochete in the blood can be made within 48 to 72 hours, if the culture tubes are placed at 30–37°C. The search for the organism should be made within the aerobic zone immediately below the surface (1.0 to 1.5 cm.), because, according to the experience of the writer, the organism is an obligatory aerobe, unable to grow in the absence of oxygen. This would explain the unsuitability of a solid medium, which prevents access of air.

The use of fresh tissue, as in the case of the relapsing fever spiro-

chete, is not required for the cultivation of this organism, and is perhaps seriously detrimental in a fluid medium. Growth takes place at any temperature from 10° to 37°C., being much more rapid at higher than at lower temperatures. The growth is almost invisible in a fluid medium, but appears as a somewhat distinct haze in semi-solid medium such as described above (*a* and *b*). The appearance is not unlike the hazy, diffuse growth of various treponemata in similar media, the only difference being that the haze in the case of the latter stops abruptly a few cc. below the surface, while the present organism is unable to grow beyond a few cc. below the surface. So far, no investigator has mentioned the visible growth of the spirochete, all describing it as invisible.

#### *Virulence.*

The American strains possess an average degree of virulence, having killed guinea pigs in 9 to 12 days in the first generation in this animal. The lesions, as well as the jaundice, were severe and typical in every respect. The second passage killed the guinea pigs in a much shorter period, 6 to 8 days, the lesions being similar to those of the first passage. Again there was no fever. They seem to be running a course similar to that of the Japanese strain in attaining a higher virulence by successive passages. The virulence of the Belgian strain appears to be less readily increased, as the guinea pigs survive 9 to 10 days. This distinction in virulence may be due to the fact that the Belgian strain was brought to us in infected rats, instead of in guinea pigs, in which the Japanese strain was received.

#### *Immunity Relations.*

Reserving the experimental details for a fuller report, it may be stated briefly that the guinea pigs which had been made immune to the Japanese or Belgian strain resisted the inoculation of the liver emulsion containing large numbers of the spirochetes of American origin, while control animals succumbed to the typical infection in 6 to 8 days. The blood sera from guinea pigs and rabbits immunized with the Japanese or Belgian strain agglutinated the American strain as strongly as they did their own strains. The immune sera exerted

germicide or lytic action upon the three strains indiscriminately. Whether or not protection experiments in guinea pigs will show any distinctions between the Belgian and the Japanese strains on the one hand and the American on the other will shortly be determined. As far as the former are concerned, the active immunity developed in guinea pigs or rabbits and their immune sera are effective, the one against the other, in almost equal titers.

The finding of the causative organism of infectious jaundice among wild rats in America, and the identification of this strain with those found in Asia and Europe seem to be particularly important in revealing a latent danger to which we have been constantly exposed but from which we escape as long as sanitary conditions are not disturbed by untoward events.

#### SUMMARY.

The principal points brought out in the present article are the following.

1. Wild rats captured in this country carry in their kidneys a spirochete which possesses the morphological and pathogenic properties characteristic of *Spirochæta icterohæmorrhagiæ* discovered by Inada in the Japanese form of infectious jaundice.

2. Cultures of the American, Belgian, and Japanese strains of the spirochete were obtained by a special technique described, the first two strains having been cultivated artificially for the first time.

3. Animals actively immunized against the Japanese strain resist inoculation, not only of the same strain, but also of the Belgian and American strains. The Belgian strain produces immunity equally effective against all three strains. Experiments to ascertain whether the immunity afforded by the American strain also protects against the Japanese and Belgian strains are in progress.

4. These findings warrant the conclusion that the spirochetes designated here as the Japanese, Belgian, and American strains are probably identical.

5. On account of its distinctive features, a new genus, *Leptospira*, has been suggested as the designation of this organism.



# IMMUNOLOGICAL STUDIES ON PURE CULTURES OF VARIOUS SPIROCHETES.

BY HIDEYO NOGUCHI, M.D., AND SEINAI AKATSU, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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## INTRODUCTION.

As a sequel to the successful cultivation of *Treponema pallidum* and other allied forms of spirochetes,<sup>1</sup> an era of test-tube experimentation on the question of immunity in syphilis has been inaugurated. Previous to the time when these organisms were obtained in pure cultures, extensive experiments were carried out with suitable animals by various investigators, notably by Metchnikoff and Roux,<sup>2</sup> Neisser and his associates,<sup>3</sup> Landsteiner and Finger,<sup>4</sup> Uhlenhuth and Mulzer,<sup>5</sup> and others. Summing up their results, we are confronted with the fact that in syphilis no immunity, in the sense generally understood in bacterial infections, is demonstrable either in animals or in man.<sup>6,7</sup> The insusceptibility of animals or human beings who have once contracted the infection and have since been apparently cured of a subsequent infection with *Treponema pallidum*, a fact well recognized since the time of Ricord,<sup>8</sup> is no longer regarded as a state of immunity, but of an anergy,<sup>3</sup> which means that the same organism is not capable of responding to another infection as long as there is a preexisting infection. Moreover, clinical and experimental data show that when an individual is cured of an attack, he soon regains practically his original susceptibility to a second

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<sup>1</sup> Noguchi, H., *Münch. med. Woch.*, 1911, lviii, 1550; *J. Exp. Med.*, 1911, xiv, 99; 1912, xv, 81, 90, 466; xvi, 194.

<sup>2</sup> Metchnikoff, E., and Roux, E., *Ann. Inst. Pasteur*, 1903, xvii, 809; 1904, xviii, 1, 657; 1905, xix, 673; 1906, xx, 785.

<sup>3</sup> Neisser, A., *Beiträge zur Pathologie und Therapie der Syphilis*, Berlin, 1911; also *Arb. k. Gsndtsamte.*, 1911, xxxvii, 569; *Deutsch. med. Woch.*, 1906, xxxii, 1, 97.

<sup>4</sup> Landsteiner and Finger, *Centr. Bakteriöl., Ite Abt., Ref.*, 1906, xxxviii, Beil., 107. Landsteiner, K., *Centr. Bakteriöl., Ite Abt., Ref.*, 1908, xli, 785.

<sup>5</sup> Uhlenhuth, P., and Mulzer, P., *Arb. k. Gsndtsamte.*, 1913, xlv, 307.

<sup>6</sup> Zinsser, H., *J. Lab. and Clin. Med.*, 1916, i, 785.

<sup>7</sup> Levaditi, C., *Z. Immunitätsforsch., Ref.*, 1910, ii, 277-318.

<sup>8</sup> Ricord, P., *Traité de la syphilis*, Paris, 1845.



infection.<sup>9</sup> In this respect, syphilis bears more resemblance to a protozoan than to a bacterial disease. Attempts to study the immunity question *in vitro* have not been lacking, and numerous investigators claim to have observed diverse specific immunity reactions, such as precipitation,<sup>10</sup> immobilization,<sup>11</sup> complement fixation,<sup>12, 13</sup> etc., when the serum or cerebrospinal fluid had been brought in contact with a material containing the treponemata of syphilis. But analysis of these observations proved that many of these phenomena could not be confirmed, and none was strictly specific. The Wassermann reaction is very constant in syphilis, but not specific. It is evident, therefore, that there is no recognized specific antigen-antibody reaction in syphilis. Schereschewsky<sup>14</sup> tried agglutination tests with his impure cultures of a spirochete derived from syphilitic material, with inconclusive results. We cannot, however, accept the negative findings of earlier investigators as final until it has been demonstrated that the techniques employed cannot be further improved. The great obstacle in the way of satisfactorily testing immune reactions in syphilis lies in the fact that a sufficient quantity of virulent organisms free from tissue constituents is obtained only with difficulty. A pure culture would fill this requirement.

In 1912 Noguchi<sup>15</sup> had already commenced to employ his several strains of culture *pallidum* for the purpose of studying the various immunity problems which were awaiting solution by means of a culture material. The main efforts were directed to finding out whether a rabbit repeatedly inoculated with the pure *pallidum* cultures will become resistant to a subsequent inoculation with a virulent *pallidum*. The cultures used were already avirulent. For comparison, another series of rabbits was similarly treated with live and killed virulent *pallida* for the same length of time, which covered a period of 5 months. The mode of immunization consisted in intravenous and intratesticular inoculation, except in the case of the live virulent material, which could not be used intratesticularly on account of its tendency to start the infection in the organ. The aim of the intratesticular mode of immunization was to find out whether there is such a thing as a local immunity in syphilis. The immunized animals were tested with a virulent strain by inoculating it into their testes. It was found that six out of the twelve rabbits immunized with the culture *pallidum* intravenously took the inoculation, while five of the twelve rabbits receiving the virulent *pallidum* took. Those which were immunized with the *pallidum* emulsions (live culture and killed

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<sup>9</sup> John, F., *Samml. klin. Vortr.*, 1909, 559 ff.

<sup>10</sup> Fornet, W., *Berl. klin. Woch.*, 1908, xlv, 85.

<sup>11</sup> Hoffmann, E., and von Prowazek, S., *Centr. Bakteriол., Ite Abt., Orig.*, 1906, xli, 741, 817.

<sup>12</sup> Detre, L., *Wien. klin. Woch.*, 1906, xix, 619.

<sup>13</sup> Wassermann, A., Neisser, A., and Bruck, C., *Deutsch. med. Woch.*, 1906, xxxii, 745.

<sup>14</sup> Schereschewsky, *Deutsch. med. Woch.*, 1909, xxxv, 1652.

<sup>15</sup> Noguchi, *J. Am. Med. Assn.*, 1912, lviii, 1163.

virulent strains) lost much of the glandular structure of their testes, some becoming mere strands of hard connective tissue. Positive takes were recorded in three of the six rabbits treated with the culture and two of the six treated with the killed *pallidum* from a syphilitic orchitis of the rabbit. As the number of animals was so small it was impossible to draw any conclusion. The results as compared with control series with normal rabbits were striking, since the takes in the normal animals were practically 90 per cent. There was an indication that the repeated inoculation of the rabbit with the *pallidum* material reduced susceptibility to a certain extent, although the difference may have been due to the altered structure of the inoculated testes. The immune sera obtained from these rabbits were also tested for agglutination, spirochetolysis, complement deviation, and opsonic property against their homologous and cross antigens. The results were at first encouraging, but repeated experiments soon showed them to be indecisive on account of the technical difficulties in the way of obtaining satisfactory antigens. This was so even with the culture *pallidum*, which at that period was either very difficult to obtain free of culture media or underwent spontaneous agglomeration.

Craig and Nichols,<sup>16</sup> employing alcoholic extracts of pure cultures of the *pallidum*, *pertenuis*, and *microdentium*, furnished by Noguchi, reported that these antigens fixed complement with syphilitic sera very much as does an alcoholic extract of a congenitally luetic fetal liver.

Kolmer,<sup>17</sup> in the meanwhile, employing a strain of culture *pallidum* furnished by Noguchi, prepared immune sera in the rabbit and demonstrated the presence of agglutinins for the strain used. His most powerful serum agglutinated the *pallidum* in a dilution of 1:1,280. Kolmer, Williams, and Laubaugh<sup>18</sup> next studied a series of human and animal sera with regard to complement fixation, with the culture *pallidum* as antigen. Their positive findings, although more numerous, were nevertheless similar to those already obtained by Noguchi.<sup>15</sup> On the other hand, they noticed that rabbit immune sera not only fixed complement with their homologous antigens, but also with washed typhoid and cholera antigens. They point out, as was previously emphasized by Noguchi,<sup>19</sup> that these immune sera do not fix complement with the alcoholic extract of the culture *pallidum*.

Kissmeyer,<sup>20</sup> employing a strain of culture *pallidum* as antigen in the agglutination tests with human sera, obtained a consistent result when compared with the clinical and Wassermann reactions, but this apparent specificity was evidently

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<sup>16</sup> Craig, C. F., and Nichols, H. J., *J. Exp. Med.*, 1912, xvi, 336.

<sup>17</sup> Kolmer, J. A., *J. Exp. Med.*, 1913, xviii, 18.

<sup>18</sup> Kolmer, J. A., Williams, W. W., and Laubaugh, E. E., *J. Med. Research*, 1913, xxviii, 345.

<sup>19</sup> Noguchi, *J. Exp. Med.*, 1909, xi, 84.

<sup>20</sup> Kissmeyer, A., *Deutsch. med. Woch.*, 1915, xli, 306.

not confirmed by Zinsser, Hopkins, and McBurney.<sup>21</sup> The recent contributions made by these investigators<sup>22</sup> have advanced our knowledge of the immunity phenomena in connection with the cultivated avirulent and uncultivated virulent *pallidum* strains; comparing the behavior of both towards the immune sera prepared by means of the former. They came to the conclusion that the immune serum behaves in the same manner as a bacterial immune serum when tested upon the culture *pallidum*; that is, it produces agglutination, causes destruction of the organism with the cooperation of fresh complement, and fixes complement with the culture antigen. On the other hand, no immunity phenomena are demonstrated when the immune serum and the virulent uncultivated organisms of the same strain are brought together. They have not mentioned, however, whether or not an immune serum prepared by means of injecting the virulent material repeatedly will ever produce a protective or spirocheticidal principle.

The work of Kolmer and Zinsser, Hopkins, and McBurney, to which we have referred, has brought out a number of facts, particularly with regard to the relation between the avirulent culture and the virulent tissue *pallidum* strains, but the question is still far from being solved. It is not superfluous, therefore, that any data bearing upon this phase of the study should be published.

The present paper deals with the results of immunological studies which had been interrupted but were recently resumed. As will be seen from the following account, we have used a considerably larger number of culture spirochetes and of animals and continued the immunization longer than any worker has previously reported. We have purposely avoided touching the question of the relation between the cultivated and uncultivated strains, as we expect to consider that point in a future paper.

#### EXPERIMENTAL.

##### *Material and Scope of Experiments.*

In the present series of immunization experiments, rabbits of about 3 kilos were employed. Several rabbits were used for each of the four strains of *Treponema pallidum*, and several also for each of the following: *Treponema calligyrum*, *Spirochæta refringens*, *Treponema microdentium*, and *Treponema mucosum*. The mode of immunization

<sup>21</sup> Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1916, xxiv, 561.

<sup>22</sup> Zinsser, H., and Hopkins, J. G., *J. Exp. Med.*, 1915, xxi, 576; 1916, xxiii, 323. Zinsser, Hopkins, and McBurney, *ibid.*, 1916, xxiii, 341.

consisted in the intravenous injection every week of 1.5 to 3 cc. of the fluid culture of each strain, the strains having been grown in a medium consisting of equal parts of normal rabbit serum and faintly alkaline bouillon, with a piece of fresh rabbit kidney. The ascitic fluid usually used was replaced by rabbit serum in order to avoid the production of a specific precipitin or of complement-binding antibodies for human proteins in the animals immunized against the spirochetal cultures. The organisms grew fairly well in this medium, owing undoubtedly to their gradual adaptation previously to various culture media which originally were unsuitable for their growth. The age of the cultures varied from 14 to 30 days, and the number of spirochetes was approximately thirty to forty per field when examined under the dark-field microscope (Leitz oc. 3,  $\frac{1}{12}$  oil immersion).

The aim of these immunization experiments was to study the development of such agglutinins, complement-binding antibodies, opsonins, and spirocheticidal principles as may be demonstrated *in vitro* in the sera of rabbits repeatedly inoculated with spirochetes. The immunity reactions were first tested with the specific organisms and then with those not used for the production of the immune sera in question. The cross examinations, various non-specific as well as

TABLE I.  
*Immunization Tests with Treponema pallidum.*

Rabbit No.	<i>T. pallidum.</i>	Period of immunization.	No. of injections.
		1915	
1	Strain McD.	Sept. 14-Dec. 17	13
2	" "	" 14- " 17	13
3	" "	" 10- " 17	11
4	" "	Oct. 20- " 17	8
5	Strain XI.	Sept. 14-Dec. 17	13
6	" XI.	" 14- " 17	13
7	" XI.	" 30- " 17	11
8	" XI.	" 30- " 17	11
9	Strain B29.	Sept. 14-Dec. 17	13
10	" B29.	" 30- " 17	11
11	Strain Z. A.	Sept. 14-Dec. 17	13
12	" "	" 14- " 17	13

the specific antigens being used against a given immune serum, were undertaken with a view to establishing the relation which may exist between different members of the group of spirochetes.

The protocols of immunization are given in Table I.

The results of experiments with the immune sera may be summarized under separate heads.

### *Agglutinins.*

The mode of determining the content of agglutinins in each serum was conducted in the following manner.

Into a series of small sterile test-tubes were measured quantities of the immune serum in amounts graduated from 0.1 to 0.000001 cc. for each tube. The distribution of the various amounts was carried out as is usual in such procedures; namely, by adequately diluting the serum with a 0.9 per cent saline solution and then taking out such quantities of each dilution as are required for titration. To each of the tubes containing various quantities of the serum was added 0.1 cc. of the spirochetal emulsion (as antigen), and the final volume was brought up to 1.5 cc. by adding the necessary quantities of 0.9 per cent saline solution to each tube. The content of the tubes was thoroughly mixed by shaking and the tubes were placed in a water incubator at 37°C. At least two tubes containing the spirochetal emulsion alone were prepared at the same time and served as controls. Concentrated and washed suspensions of various spirochetes derived from pure cultures in a fluid medium were used as antigens. 0.1 cc. of the suspension of each strain in 0.9 per cent saline solution was used for each tube. The number of spirochetes varied in different suspensions, but there were over 100 per field (Leitz oc. 3,  $\frac{1}{2}$  oil immersion, dark-field illumination), and the addition of 0.1 cc. of the antigen emulsion to each tube (total volume of fluid, 1.5 cc.) produced grayish white, semitranslucent turbidity. The turbidity in the control tubes gradually subsided while standing in the incubator, but was never completely cleared up, even after 24 hours' standing. Readings of the results were made twice, once after 2 hours' incubation, and again at the end of 24 hours at a temperature of 15°C. The sera were used without any modification, such as inactivation at 56°C.

As may be seen from Table II, we first tested the agglutinating powers of various immune sera for their homologous strains; that is, for the strains which had been used for producing them in the rabbits by repeated intravenous injections. Thus, eleven sera were titrated with four different strains of the *pallidum*, two to four immune sera for each strain. As in the case of the *pallidum* the remaining



immune sera, eight in number, were tested with their homologous species, the *calligyrum*, *refringens*, *microdentium*, and *mucosum*, which had been employed for their production.

It may be mentioned that we had to make a number of preliminary experiments before we felt assured of obtaining fairly uniform and reliable results. The reading of a strong agglutination was quite easy, as the organisms rapidly settled down, and the sediment adhered firmly to the side and bottom of the tube, but the less intense reaction was not as clear as we wished; hence, our reading of the minimum zone of the reaction was more or less arbitrary. The titers of agglutinins in different sera read after 2 hours' incubation were somewhat lower than those recorded after the same set of tubes had been left at room temperature for 24 hours longer. It was always necessary, in order to detect a slight degree of agglutination, to shake up the sediment and compare the granulation or clumping of the suspension with a control tube without any serum. We have resorted solely to the macroscopic reading of the reaction. A microscopic examination of the sediment adhering rather firmly to the side and bottom of the test-tubes where a definite agglutination occurred revealed enormous masses of entangled spirochetes, some apparently undergoing morphological modifications, as shown by a granular appearance or by relaxation of curves.

In Table II several facts seem to stand out conspicuously. In the first place, the titers of agglutinins developed in the rabbits treated with the saprophytic species, namely, the *calligyrum*, *refringens*, *microdentium*, and *mucosum*, are decidedly lower than those found in the sera produced by immunizing the rabbits with the *pallidum*. Secondly, the amounts of agglutinins in different immune sera are not in direct proportion to the number of injections or the duration of the immunization. Thus, the highest titer was found in Serum 4, in which 0.00001 cc. caused a definite, and 0.000025 cc. a slight agglutination, while Serum 9, notwithstanding the fact that the animal had five more injections than the former, required 0.0025 cc. for a definite reaction. Again, it is evident, with regard to the production of the agglutinins, that different rabbits react differently to the same spirochetal antigens. The apparent difference between the titers of the *pallidum* group and those of the other group requires an ex-



TABLE II.

*Tubes Incubated for 2 Hours in the Water Bath. Antigen: 0.1 Cc. of Emulsion of Fluid Cultures 2 Months Old. Total Volume 1.5 Cc.*

Antigens.	Immune sera.	Titers of agglutinins.
		cc.
<i>T. pallidum</i> , Strain McD.....	No. 2	0.000025
	" 3	0.0001
	" 4	0.00000375
" XI.....	" 5	0.00005
	" 6	0.0005
	" 7	0.00004
	" 8	0.000025
" B29.....	" 9	0.001
	" 10	0.00005
" Z. A.....	" 11	0.001
	" 12	0.000025
<i>T. calligyrum</i> .....	" 13	0.0005
	" 14	0.001
<i>S. refringens</i> .....	" 15	0.00125
	" 17	0.001
<i>T. microdentium</i> .....	" 18	0.0025
	" 19	0.001
<i>T. mucosum</i> .....	" 20	0.001
	" 21	0.01

planation. Whether the phenomenon is due to the greater amounts of agglutinins produced in the rabbits immunized with the *pallidum* or to a physical or possibly also chemical factor inherent in the *pallidum* antigen has not been established. It is also possible that our results with the saprophytic varieties happened to be inferior merely because the animals used were unfavorable individuals. The results might have been different if we had employed more animals.

Of more interest are the results of cross examinations of various immune sera with regard to their specificity towards the homologous and heterologous antigens. Eight immune sera were chosen to be tested, each with six strains of *Treponema pallidum* and one of *Treponema calligyrum*, *Spirochata refringens*, *Treponema microdentium*, and *Treponema mucosum*, or ten different antigens in all. Stated in more detail, the procedure was as follows:

Serum 4 was produced by injecting the animal with Strain McD. of the *pallidum* and was tested not only with the emulsion of the same strain (homologous), but also with those of the Strains XI, R, C<sub>2</sub>, B30, and Z. A. of the *pallidum*, as well as with those of the *calligyrum*, *refringens*, *microdentium* and *mucosum* (heterologous and of different species). Serum 8, produced with Strain XI, and Serum 12, produced with Strain Z. A. of the *pallidum*, were likewise tested against their homologous as well as their heterologous antigens, while Serum 10, the homologous antigen of which was Strain B29, was tested with all ten heterologous antigens, since there was no homologous one in the series. Sera 14 and 17 of the *calligyrum* and *refringens* of the non-pathogenic spirochetes of the genitalia, and Sera 18 and 20 of the *microdentium* and *mucosum* of the buccal cavity were each tested with one homologous and nine heterologous antigens. In order to secure as closely comparable results as possible, all the tests were carried out in parallel series on the same day and with the same materials. The first reading was recorded after completion of the 2 hour incubation in the water thermostat and the second at the end of the 24 hour period at room temperature. The latter readings are given in Table III.

In the analysis of Table III, several points are brought out. In the first place, there is a pronounced degree of specificity of each characteristic for its homologous group of antigens. Thus, the immune sera belonging to the *pallidum* group agglutinated most strongly when brought together with the emulsion of the *pallidum* strains, but not at all when mixed with the *microdentium* or *mucosum*. The reverse is also true; that is, the immune serum produced by means of the *microdentium* agglutinated none of the other groups, except for a slight reaction with the *mucosum*. The *mucosum* immune serum showed its strongest action on the *mucosum* antigen, although there was a more or less feeble reaction with some of the other spirochetes, especially the *microdentium*. The relation between the *calligyrum* and *refringens* seems very close, considered from the point of view of the agglutination reaction. They did agglutinate mutually to such an extent that they might be included in the same group. As already mentioned above, they showed no affinity whatever for the group of the mouth spirochetes. On the other hand, there existed a certain degree of so called group reaction between the *pallidum* and the *calligyrum* groups. It may be pointed out that in spite of the close relation between the *calligyrum* and the *refringens*, the immune sera pertaining to the *pallidum* did not noticeably agglutinate the *refringens*, while the same sera invariably agglutinated the *calligyrum*.

TABLE III.

*Specificity of Immune Sera towards the Homologous and Heterologous Antigens.*  
*Results of Incubation for 24 Hours at Room Temperature.*

Antigens.	Immune sera.							
	No. 4 homologous with Strain McD.	No. 8 homologous with Strain XI.	No. 10 homologous with Strain B29.	No. 12 homologous with Strain Z. A.	No. 14 homologous with <i>T. calligyrum</i> .	No. 17 homologous with <i>S. refringens</i> .	No. 18 homologous with <i>T. microdentium</i> .	No. 20 homologous with <i>T. mucosum</i> .
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
<i>T. pallidum</i> .								
Strain McD.....	0.0005	0.0005	0.0005	0.0025	0.05	Trace at 0.05.	None at 0.05.	None at 0.05.
“ XI.....	0.0005	0.0005	0.0005	0.005	0.05	Trace at 0.05.	None at 0.05.	None at 0.05.
“ R.....	0.0005	0.005	0.005	0.005	0.05	0.05	None at 0.05.	Trace at 0.05.
“ C <sub>2</sub> .....	0.0005	0.001	0.0005	0.001	0.01	Trace at 0.05.	None at 0.05.	Trace at 0.05.
“ B30.....	0.0005	0.0025	0.0005	0.005	0.05	Trace at 0.05.	None at 0.05.	Trace at 0.05.
“ Z. A.....	0.0005	0.0005	0.0005	0.0005	0.05	0.05	None at 0.05.	Trace at 0.05.
<i>T. calligyrum</i> .....	0.05	0.05	0.025	0.025	0.0005	0.0075	None at 0.05.	Trace at 0.05.
<i>S. refringens</i> .....	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	0.005	0.0025	None at 0.05.	Trace at 0.05.
<i>T. microdentium</i> ...	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	None.	0.001	0.01
<i>T. mucosum</i> .....	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	None at 0.05.	“	0.05	0.001

in doses between 0.05 and 0.025 cc., that is, in a dilution of 1:20 to 1:40. This group reaction is, of course, far below the titers of these sera for the *pallidum* antigens, in which they varied from 0.005 (1:200) to 0.0005 cc. (1:2,000), and still further down to 0.00001 cc. (1:100,000) (Table II). Conversely, the *calligyrum* serum, which agglutinated its own antigen in a dose of 0.0005 cc. (1:2,000) produced a group reaction with the *pallidum* emulsions in doses ranging from

0.05 (1:20) to 0.01 cc. (1:100). In this respect, the behavior of the *refringens* serum was somewhat similar to that of the *calligyrum*, as it also produced a distinct agglutination with some of the *pallidum* antigens when employed in a concentration stronger than 1:20, sometimes even in a dilution of 1:40.

As to the results obtained among the *pallidum* antigens with the corresponding and cross immune sera, it will be noticed that the reaction between the homologous antigens and immune sera are, as a rule, stronger than those which occurred when the former were mixed with the sera produced by the other strains. On the other hand, a serum which strongly agglutinates a certain strain or strains may not necessarily agglutinate others, while the latter may yet be the most readily and strongly agglutinated by another serum. In other words, our present study indicates that the agglutinin titers of these sera are variable according to the differences in the individual strains of the same group. This phenomenon has long been recognized in the agglutination of various bacteria by their immune sera, and apparently it holds good in the case of the spirochetes. The complex composition of agglutinins, such as partial agglutinins of different affinities or still only scantily understood factors in agglutination reactions, is accountable for the intricacy of the so called specific as well as group reactions.

#### *Influence of Time upon the Agglutinins in Vitro and in Vivo.*

The immune sera used in the foregoing experiments were preserved in a refrigerator at the temperature of 6°C. for 3 months and then examined for their strength. In the meantime, the rabbits immunized were not given any further injection of the spirochetal emulsions for the same length of time, and then their sera were drawn for the titration of agglutinins. Table IV shows the titers of these sera as compared with their original strength.

The rates with which the agglutinin contents of the immune sera lost strength during the 3 months seem to be irregular and show no constant proportion to the original titers of the sera. There is, however, a general tendency of the agglutinins to disappear from the serum more rapidly *in vivo* than *in vitro*. The titers of the ag-

TABLE IV.

*Effect of Time upon Agglutinins in Vitro and in Vivo.*

Immune sera.	Titer		
	Original.	After 3 mos. kept at 6°C.	After 3 mos. in the animal.
	cc.	cc.	cc.
No. 2, <i>T. pallidum</i> , Strain McD.....	0.000025	0.00025	0.001
" 8, <i>T.</i> " " XI.....	0.000025	0.0005	0.001
" 10, <i>T.</i> " " B29.....	0.00005	0.0001	0.001
" 12, <i>T.</i> " " Z. A.....	0.000025	0.0001	0.001
" 14, <i>T. colligyrum</i> .....	0.001	0.01	0.1
" 15, <i>S. refringens</i> .....	0.00125	0.01	0.1
" 19, <i>T. microdentium</i> .....	0.001	0.01	<0.1
" 20, <i>T. mucosum</i> .....	0.001	0.01	<0.1

glutinin in the sera derived from these rabbits at the end of 3 months after discontinuation of immunization were reduced to about one-one hundredth in Nos. 14, 15, 19, 20; one-fortieth in Nos. 2, 8, 12; and one-twentieth in No. 10, while the original sera when preserved in the refrigerator during the same period of time lost their strength down to about one-tenth of the initial titers, except in No. 8, where it was almost twice as much weakened as in the rest. It may therefore be assumed that during the first 3 months after the cessation of immunization, the disappearance of the agglutinins for various spirochetes was almost ten times as fast in the animal body as in the test-tubes kept at a temperature of 6°C.

#### *Complement Fixation.*

The immune sera were also tested for their property of binding complement when mixed with their homologous as well as heterologous antigens. The technique of carrying out the tests was that usually employed in such experiments.

The immune sera were inactivated at 56°C. for half an hour before use. The antigens were prepared from the pure cultures of various spirochetes grown in a fluid medium consisting of equal parts of rabbit serum and slightly alkaline bouillon, with a piece of normal rabbit kidney. The spirochetes were collected from cultures which had been vigorously growing for 4 weeks. For the purpose of concentration and purification, the spirochetes were collected by a prolonged



centrifugation and washed again in 0.9 per cent saline solution. The sediment, re-suspended in an adequate volume of 0.9 per cent saline solution, showing about twenty to thirty spirochetes under dark-field illumination, was used as the antigen. The relative quantities of different factors were as follows: The immune serum in quantities of from 0.1 to 0.0001 cc., complement 0.04 cc. (2 units), antigen 0.2 cc., sheep corpuscles 5 per cent, anti-sheep amboceptor 0.0002 cc. (corresponding to 2 units), total volume made to 1.5 cc. with 0.9 per cent saline solution. The first incubation was for 1 hour (at 36°C. in the water bath), and the second also for 1 hour. The quantities of immune serum and the spirochetal suspensions chosen for the above experiments were previously tested and found to be not anticomplementary in themselves. The results were recorded after allowing the tubes to stand for 2 hours longer at room temperature.

TABLE V.  
*Complement Fixation with Homologous Antigens.*

Antigens 0.2 cc.	Immune sera.	Titers of fixation.
		cc.
<i>T. pallidum.</i>		
Strain McD.....	No. 2	0.005
	" 3	0.002
	" 4	0.005
" XI.....	" 5	0.005
	" 6	0.1
	" 7	0.005
	" 8	0.008
" B29.....	" 9	0.007
	" 10	0.003
" Z. A.....	" 11	0.002
	" 12	0.0003
<i>T. calligyrum.</i> .....	" 14	0.01
<i>S. refringens.</i> .....	" 15	0.1
	" 17	0.03
<i>T. microdentium.</i> .....	" 18	0.007
	" 19	0.05
<i>T. mucosum.</i> .....	" 20	0.002
	" 21	0.005

Table V shows that the titers of the immune sera derived from different rabbits vary considerably according to the individual variations that exist among the animals. For example, Immune Sera 6 and 15 showed an extremely low power for complement fixation, in spite of the fact that the rabbits in the same groups, particularly



in the Strain XI series, produced fairly high titers. The titers of the *pallidum* group were in the main quite high, while the *calligyrum* and *microdentium* remained much less developed. It is interesting to note that of the two animals treated with the *mucosum*, both responded with high titers. When compared with the agglutinin titers of the same sera, the fixation titers are much lower but seem to run fairly parallel, in the sense that a serum with stronger agglutinating power had also a higher titer for complement fixation. There were a few exceptions to this rule.

For determining the question of specificity of the complement fixation reaction with the spirochetes a series of experiments was performed in which a certain number of the immune sera were tested, each in turn, with the entire set of antigens. Table VI gives the results. The technique used was the same as before.

Table VI shows that various immune sera fixed complement more strongly with their corresponding antigens than with a heterologous one. Among the *pallidum* strains, the reaction is fairly interchangeable, although some immune sera (Nos. 3 and 7) have caused only a weak fixation with a certain antigen (Strain B29). The *calligyrum* serum produced a marked fixation, not only with its own antigen, but also with certain *pallidum* strains (Strains XI and Z. A.) to such an extent that in a dilution of 1:30 no difference could be distinguished between the *calligyrum* and these two *pallidum* strains. In this connection, mention may be made of an analogous instance where one of the *pallidum* immune sera (No. 3) fixed complement with the *calligyrum* antigen distinctly in a dilution of 1:30. The *refringens* serum fixed complement best with its own antigen, but also feebly with all the other antigens except that of the *mucosum*. A similar group reaction was evident with the *calligyrum* and *mucosum* sera. In spite of the presence of a certain amount of group reaction, the specific character of the complement fixation phenomenon seems to hold. At least, the differences between the *pallidum*, *refringens*, *microdentium*, and *mucosum* are sufficient for considering the reaction specific. The *calligyrum* showed much affinity for the *pallidum* on the one hand and for the *refringens* on the other.

TABLE VI.

*Complement Fixation with Heterologous Antigens.*

Immune sera.		Antigens used for cross titration.							
		<i>T. pallidum</i> strain.				<i>T. calli- gyrum.</i>	<i>S. re- fringens.</i>	<i>T. mic- roden- tium.</i>	<i>T. mu- cosum.</i>
		McD.	XI.	B29.	Z. A.				
	cc.								
No. 3 homologous with Strain McD.	0.1	++	++	<+	++	+	—	—	< <+
	0.03	++	++	—	++	<+	—	—	—
	0.01	+	+	—	++	—	—	—	—
No. 7 homologous with Strain XI.	0.1	+	++	++	++	+	—	—	—
	0.03	+	++	<+	+	—	—	—	—
	0.01	<+	+	—	<+	—	—	—	—
No. 10 homologous with Strain B29.	0.1	++	++	++	++	<+	—	—	—
	0.03	++	++	++	++	—	—	—	—
	0.01	++	+	++	++	—	—	—	—
No. 11 homologous with Strain Z. A.	0.1	+	++	++	++	<+	<+	—	—
	0.03	<+	+	+	++	—	—	—	—
	0.01	—	<+	—	++	—	—	—	—
No. 14 homologous with <i>T. calligyrum</i> .	0.1	< <+	++	<+	+	++	<+	< <+	< <+
	0.03	—	<+	—	<+	<+	—	—	—
	0.01	—	—	—	—	< <+	—	—	—
No. 17 homologous with <i>S. refringens</i> .	0.1	<+	+	<+	<+	+	+	< <+	—
	0.03	—	—	—	—	< <+	< <+	—	—
	0.01	—	—	—	—	—	—	—	—
No. 18 homologous with <i>T. microden- tium</i> .	0.1	—	—	—	<+	< <+	—	++	<+
	0.03	—	—	—	—	—	—	++	—
	0.01	—	—	—	—	—	—	<+	—
No. 21 homologous with <i>T. mucosum</i> .	0.1	<+	<+	<+	<+	< <+	—	<+	++
	0.03	—	—	—	—	—	—	—	++
	0.01	—	—	—	—	—	—	—	++

*Spirocheticidal Properties.*

In order to determine whether or not these immune sera have a destructive power upon the spirochetes *in vitro*, several series of tests were carried out. Because of the part played by complement in a bacteriolytic process, our experiments were so arranged that in one set the immune serum was allowed to act alone, while in another, both complement and immune serum were put together with the spirochetes.

The immune sera were inactivated at 56°C. for 30 minutes before use, and the spirochetes were from recent fluid cultures and were very active. The amount of immune serum was graduated from 0.1 down to 0.00005 cc., while spirochetes and complement were used in a quantity of 0.1 cc. each for each test-tube. The volume of the resulting fluid in each tube was made uniformly 1.1 cc. by adding 0.9 per cent saline solution. After all the factors, namely, spirochetes, immune serum, and complement, had been mixed, the tubes were placed in a water bath incubator at 37°C. for 1 hour, and then the whole contents were used for cultivation in the usual ascitic agar tissue medium. The results were recorded at the end of 2 or 3 weeks of incubation at 36°C. Normal rabbit serum was also tested as control.

The results of experiments in which most of the antisera were tested show that the anti-*pallidum* Serum 12 suppressed the growth of its homologous strain Z. A. in a dose of 0.001 cc. in the presence of complement, while scanty growth was observed in all the tubes where there was no complement added to the immune serum. Normal rabbit serum had no appreciable effect on the growth of this strain. It was noted also that the number of spirochetal colonies diminished almost proportionately with the gradual increase of the specific immune sera, until, in quantities of serum beyond a certain limit, no growth was observed. With the anti-*pallidum* Serum 4, and possibly also No. 8, the titer was 0.0003 cc. It was not rare for the anti-*pallidum* sera to attain the titer of 0.001 cc. On the other hand, the immune sera for the *refringens*, *calligyrum*, and *mucosum* were far inferior in their restraining influence upon their homologous species, none of them being strong enough to inhibit the growth in a dose below 0.01 cc. In the *mucosum*, 0.01 cc. of the antiserum failed to kill the organism.

In parallel series of experiments without the addition of complement, it was found that the powerful immune sera (Nos. 4 and 8) caused a considerable restraint upon the growth of the homologous strains, the only difference between these sera and those containing complement being that their effects are less marked. When used in quantities below 0.03 cc., neither of these sera caused a complete suppression of the growth of organisms acted upon by them. The question naturally presents itself as to whether the suppression of growth in cases where complement and the immune sera were employed was due to the actual destruction of the organisms or merely

to the agglutination. The latter assumption may be dismissed, as the phenomenon does not occur when the immune sera alone (containing the agglutinins) are used. Of course, the sparseness of colonies in these tubes must be partly due to the agglutination, which tends to clump many thousand spirochetes to start a single colony. Yet a total suppression cannot be explained on this ground, since a single colony, once it has begun to grow, will finally form a diffuse growth throughout the medium. As will be described later, the failure of the spirochetes to grow after being subjected to the action of complement and immune serum was largely due to the actual destruction of the spirochetes. Syphilitic rabbit serum was also tested and showed that there was an unmistakable destruction of the *pallidum* strain in quantities above 0.03 cc. Normal rabbit serum showed a slight restraint upon growth of this strain when used in doses of 0.1 cc. Neither of these sera had any ill effect upon the spirochetes when complement was omitted from the mixture.

*Microscopic Observations on the Effect of the Immune Sera upon Spirochetes in Vitro.*

Immune Sera 4 (Strain McD.), 8 (Strain XI), 10 (Strain B29), and 12 (Strain Z. A.) were chosen for a series of observations in which we followed the changes which take place in *Treponema pallidum* when the organisms have been subjected to the action of these sera. Three strains of the *pallidum*, McD., R, and Z. A., were used for the purpose. As in the previous experiments, the action of the immune sera was studied with and without the addition of complement (guinea pig fresh serum).

The sera were inactivated at 56°C. for 30 minutes. The mixture of the serum, spirochetal suspension (McD.), and complement (in cases where it was added) was incubated in a water bath at 37°C. for 3 hours, and microscopic examinations were made during and after incubation. Similar results were obtained with the strains of R and Z. A.

Table VII demonstrates the fact that *Treponema pallidum* undergoes a fundamental change in its morphology when acted upon by its immune sera and complement at an optimum temperature. The phenomenon is similar to the dissolution or disintegration of various

TABLE VII.

*Effect of Immune Sera upon Spirochetes in Vitro.*

Strain McD. 0.1 cc. + Immune Serum 4 (Strain McD.) 0.1 cc. + complement 0.1 cc.	The organisms were strongly agglutinated within 1 hr., then they became granular in appearance, and only a few retained their form.
Strain McD. 0.1 cc. + Immune Serum 8 (Strain XI) 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + Immune Serum 10 (Strain B29) 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + Immune Serum 12 (Strain Z. A.) 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + Immune Serum 4 (Strain McD.) 0.1 cc. + no complement.	The agglutination was even stronger than in the above series. At the end of 24 hrs. the organisms kept their form.
Strain McD. 0.1 cc. + Immune Serum 8 (Strain XI) 0.1 cc. + no complement.	
Strain McD. 0.1 cc. + Immune Serum 10 (Strain B29) 0.1 cc. + no complement.	
Strain McD. 0.1 cc. + Immune Serum 12 (Strain Z. A.) 0.1 cc. + no complement.	
Strain McD. 0 + no serum + complement.	No noticeable changes.
" " 0 + " " + no "	

microorganisms under the influence of an immune serum. In the absence of complement, the organisms were strongly agglutinated, but not dissolved, even after 24 hours.

In the following experiment, attempts were made to study the part, if any, played by leukocytes in the destruction of the spirochetes *in vitro*. Leukocytes both from normal and from immunized rabbits were collected by Wright's method. The citrate blood was briefly centrifuged, and the leukocytic layer of the sediment carefully skimmed and put into another centrifuge tube. In ordinary opsonin work, the cells thus collected are used without washing, but in the present experiment, they were washed with a 0.9 per cent saline solution by a renewed centrifugalization. The immune leukocytes were obtained from a rabbit which had been immunized with the Z. A. strain (No. 12).

The technique was as follows: To 0.1 cc. of a suspension of one of the *pallidum* strains were added 0.1 cc. of the leukocytic suspension, 0.1 cc. of each of the immune sera, and 0.1 cc. of complement. The mixtures, after being well stirred,



were incubated in a water bath at 37°C. and examined at various intervals; for example, 30 minutes, 1 hour, 2 hours, and 4 hours (kept at room temperature). The examinations were made without staining under the dark-field microscope, and also as film preparations stained by Giemsa as well as by a modified Fontana silver impregnation. Films prepared from a mixture in which unwashed citrate leukocytic suspension was used washed off the slides very readily and could not be depended upon for accurate results. Normal as well as immune serum and normal leukocytes were also used (Table VIII).

TABLE VIII.

*Effect of Leukocytes upon Spirochetes in Vitro.*

Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 4, 0.1 cc. + complement 0.1 cc.	Strong agglutination and gradual lysis, many spirochetes adhering to the leukocytes, into which some were definitely ingested. Total disappearance of spirochetes in 24 hrs.
Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 8, 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 10, 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + immune leukocytes (No. 12) 0.1 cc. + Immune Serum 12, 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 4, 0.1 cc. + complement 0.1 cc.	Strong agglutination and general disintegration of spirochetes but decidedly less than in the preceding series. Phagocytosis present.
Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 8, 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 10, 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + normal leukocytes 0.1 cc. + Immune Serum 12, 0.1 cc. + complement 0.1 cc.	
Strain McD. 0.1 cc. + no leukocytes + Immune Serum 4, 0.1 cc. + complement 0.1 cc.	Much lysis in 24 hrs.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + no immune serum + complement 0.1 cc.	No striking changes.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + no immune serum + no complement.	No lysis or phagocytosis.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + normal serum 0.1 cc. + complement 0.1 cc.	No lysis or phagocytosis.
Strain McD. 0.1 cc. + immune leukocytes 0.1 cc. + normal serum 0.1 cc. + no complement.	No lysis or phagocytosis.



Parallel series of experiments were also carried out with Strains R and Z. A. with similar results. It seems apparent, therefore, that some of the spirochetes are readily taken up by immune as well as normal rabbit leukocytes, when an immune serum and complement are simultaneously added to the mixture. The number of spirochetes taken up by phagocytes is small compared with what we are accustomed to see with bacteria. It may be due to the filamentous feature of the organisms, which prevents the cells engorging them with readiness, or what appears still more probable is that they form enormous masses of entangled nets too large to be taken up by the phagocytes, and then gradual lysis occurs. It is also possible that the engorged spirochetes quickly disappear through intracellular digestion. At all events, the agglutinated masses become gradually granular and indistinct and finally undergo dissolution. The completeness with which this process proceeds is most marked when immune leukocytes are used. Perhaps there exists within these cells certain elements which render the lytic processes more energetic than when only immune serum and complement are used. In the absence of complement, the immune serum produced only a slight degree of phagocytosis and no lysis. The organisms, after being acted upon by the leukocytes in the immune serum and complement mixture, lose their property to take Giemsa stain as strongly as those not so treated. Their curves are seen to be flattened in many specimens. They still retain their affinity for the silver precipitation method (Fontana). It has been difficult to stain the spirochetes distinctly within the phagocytes, although they could be seen in fresh preparations by means of dark-field illumination. The large number of spirochetes adhering to the leukocytes prevents a clear appearance of the phenomenon.

A series of experiments was performed with normal human leukocytes in conjunction with the immune rabbit sera and complement. The results were similar to those recorded for normal rabbit leukocytes.

*Effects of Immune Sera and Leukocytes upon the Uncultivated Strain of Treponema pallidum.*

The action of the sera derived from rabbits immunized with cultivated strains of *Treponema pallidum* may also be studied. It may be tested by subjecting a suspension of a virulent testicular material rich in the *pallidum* to the effect of the immune sera and then inoculating certain animals susceptible to experimental syphilis. Another procedure would be to study the immunity phenomena which follow the mixing of the *pallidum* and the serum *in vitro*. Agglutination, immobilization, granular disintegration, dissolution, or complement binding may be studied. The first method is subject to the difficulties inherent in experiments in which the susceptibility of different individuals constitutes an inconstant factor. In fact, our preliminary experiments indicated that study with extensive material is required. This phase of the study has been under investigation by one of us since 1911 and will have to be continued for a longer time. The test-tube phenomena were better observed.

To 0.1 cc. of a rich suspension of virulent *pallidum* from syphilitic orchitis of a rabbit, 0.1 cc. of each of the several immune sera, comprising Nos. 4 (Strain McD.), 8 (Strain XI), 12 (Strain Z. A.), and 10 (Strain B29) was added, and 0.1 cc. of a 40 per cent solution of fresh guinea pig serum as complement. The mixture was made up to 1 cc. by adding a 0.9 per cent sterile saline solution and then placed in a water bath kept at the temperature of 37°C. The mixture, an opalescent fluid, was examined from time to time for the agglutination, motility, or disintegration of the *pallidum* under the dark-field microscope. Controls with the *pallidum* alone, the *pallidum* with the immune serum (inactivated), and the *pallidum* with the complement were provided.

The results of our observations were in the main similar to those obtained by Zinsser, Hopkins, and McBurney, except for a few points. It was noticed in our experiments that the particular strain of *Treponema pallidum* employed became sooner or later immobilized by the addition of the immune sera, and none was motile after 3 hours at 37°C. In the control tubes containing plain saline or complement solution, the organisms were still active for several hours longer. In some control tubes, there were a few motile *pallida* at the end of 24 hours. The immobilizing effect was augmented by the presence of complement. There was no definite agglutination or dissolution of the *pallidum* except in cases of Sera 4 and 8, where there were small clumps of entangled immobilized organisms suggesting a slight agglutination. But we were unable to find definite disintegration of the *pallidum*.

Studies were also made to discover whether or not leukocytes from an immune or normal rabbit ingested the tissue *pallidum* under the influence of the immune serum, but the results were inconstant. Although there was an unmistakable phagocytosis in the presence of the immune serum, it was slight in comparison with the cultivated strains, and there was no general disintegration of the organisms. In considering the above results, it appears as though the immune sera prepared by injecting the rabbit with avirulent strains of culture *pallidum* exerted much less effect upon a virulent strain derived from a syphiloma in a rabbit. Whether or not this is due to unsuitable immunization or to a modification in strains is not shown by the present experiments. The failure of agglutination of the tissue *pallidum* may have been due to the presence of various tissue proteins simultaneously introduced into the mixture, for these apparently indifferent substances can often interfere with agglutination or complement fixation.<sup>23</sup> The entire question as to the relation between the uncultivated virulent strains and those which had become avirulent through cultivation is still under investigation.

#### CONCLUSIONS.

Experiments were carried out for the study of culture spirochetes in their relation to various immunity reactions *in vitro*. Several strains of *Treponema pallidum* and one each of *Treponema calligyrum*, *Spirochæta refringens*, *Treponema microdentium*, and *Treponema mucosum* were used. Tests were made of immune substances responsible for agglutination, complement fixation, spirocheticidosis, and opsonization. In cases of agglutination and complement fixation, cross titrations were made.

1. In the sera derived from rabbits immunized with various spirochetes agglutinins were demonstrated in varying quantities for the homologous antigens. The amounts of agglutinins developed were considerably higher in the *pallidum* immune sera than in the other groups. There was no parallelism between the amounts of antigens injected and the amounts of agglutinins developed.

2. Cross titrations among different *pallidum* strains revealed that

<sup>23</sup> Noguchi, H., and Bronfenbrenner, J., *J. Exp. Med.*, 1911, xiii, 92.

the agglutination is not necessarily strongest when homologous antigens and immune sera are brought together.

3. On the other hand, the reactions between the immune sera and antigens belonging to different species were sufficiently specific to justify the grouping.

4. Certain degrees of group reaction were observed between the *pallidum* immune sera and the *calligyrum*, and occasionally very faintly also between the *pallidum* and the *refringens* antigens and *vice versa*. There was a much more pronounced group reaction between the *calligyrum* and *refringens*. The immune serum and antigen of the *microdentium* showed a slight affinity for the *mucosum* but none for the *pallidum*, *calligyrum*, or *refringens*, while the *mucosum* immune serum caused a slight agglutination with many members of the other groups. Hence, it appears that the *pallidum* is more or less related to the *calligyrum*, while the affinity between the *calligyrum* and *refringens*, and possibly also between the *calligyrum* and *mucosum* in a much smaller degree, seems close. The *microdentium* showed the least relation to any other spirochetes.

5. Titration of agglutinins in the sera obtained 3 months after the cessation of immunization revealed that the agglutinin contents were already greatly reduced, having fallen roughly to 0.01 of the original strength. The rates of disappearance were irregular in different animals and bore no direct relation to the initial titers. Titration made of the immune sera which had been preserved aseptically in a refrigerator (6°C.) during the same period (3 months) indicated that the original strength of these sera was reduced to about one-tenth. The agglutinins for spirochetes disappear from the rabbit's body much more rapidly than they are reduced in the separated sera by deterioration on standing at 6°C.

6. Titration of the immune sera for complement fixation power showed with a few exceptions, in which there was only slight complement binding, that the titers were high enough to indicate the presence of this principle. The anti-*pallidum* sera possessed higher average titers than the other immune sera tested with correspondingly homologous antigens. The least active were the anti-*refringens* sera.

7. Cross titration of anti-*pallidum* immune sera for complement fixation showed that a given serum with a high titer for its own strain of antigen was also strong with most of the other strains of the



*pallidum*. Instances occurred also in which the titers with heterologous *pallidum* antigens fell far below those of the homologous. Group reactions between the different spirochetes, such as the *pallidum* and the *calligyrum*, the *calligyrum* and the *refringens*, and the *microdentium* and the *mucosum*, were also indicated. The *mucosum* and the *pallidum* showed a slight degree of group reaction. No anti-*pallidum* serum fixed complement with the *microdentium*.

8. The immune sera were tested for their spirocheticidal properties *in vitro* against the correspondingly specific and heterologous varieties with and without the addition of complement. Many of the anti-*pallidum* sera killed their own strains. Normal rabbit serum exhibited only a slight degree of inhibition. Without complement, the immune sera caused a considerable reduction in the number or density of colonies, but not a complete suppression of growth. Complement alone had no injurious effect upon the *pallidum* strains. The antisera for the *calligyrum*, *refringens*, and *mucosum* showed feeble spirocheticidal action, while the antisera for *microdentium* was stronger. A syphilitic rabbit serum tested against a strain of culture *pallidum* gave a feeble inhibitory effect.

9. Under the influence of immune sera and complement, the spirochetes undergo within a few hours complete disintegration or granular degeneration. Without complement, they are more powerfully agglutinated, but no disintegration occurs, even after 20 hours, and complement alone has no effect.

10. In the presence of homologous immune serum and complement, the culture *pallidum* may be ingested by the leukocytes, but phagocytosis is slight, possibly on account of the filamentous nature of the organisms. The spirochetes in such a mixture disintegrate within a few hours, disintegration being especially rapid when the immune leukocytes are used. In the absence of immune serum, phagocytosis is not noticeable, while without complement but in the presence of immune serum and leukocytes, some phagocytosis, without subsequent lysis, occurs.

A virulent strain of *pallidum*, obtained from syphilitic orchitis in a rabbit, exposed to agglutination, lysis, and phagocytosis by an immune serum prepared by means of culture *pallidum* strains, showed only slight agglutination and phagocytosis but rapid immobilization without disintegration in the presence of complement.

## A CONTRIBUTION TO THE BACTERIOLOGY OF ACUTE ANTERIOR POLIOMYELITIS.\*

By JOHN A. KOLMER, M.D., CLAUDE P. BROWN, M.D., AND ANNA M. FREESE, M.D.

*(From the Laboratory of the Philadelphia Hospital for Contagious Diseases and the McManes Laboratory of Experimental Pathology of the University of Pennsylvania, Philadelphia.)*

PLATE 66.

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During the recent outbreak of acute anterior poliomyelitis in Philadelphia (1916) we began a series of bacteriological studies with the blood and cerebrospinal fluid collected from patients during life and with various tissues secured at eight autopsies.

Early in the work we obtained cultures of diplococci, streptococci, and other microorganisms, but on account of the consensus of opinion to the effect that these bacteria are of little or no importance in the etiology of the disease, we devoted our efforts toward the cultivation of the organism described by Flexner and Noguchi (1) in 1913. As the result of recent communications by Mathers (2), Rosenow and his associates (3), and Nuzum and Herzog (4), setting forth renewed claims for the etiologic relation of these cocci to acute anterior poliomyelitis, we have given the cultures further and more extensive study, and the purpose of this communication is to present the results of our work with many different cultures of these easily cultivated bacteria isolated from persons ill with acute anterior poliomyelitis.

\* Part of the paper was read in the symposium upon acute anterior poliomyelitis before the Philadelphia County Medical Society, November 8, 1916. This work was instituted in the laboratory of the Philadelphia Hospital for Cutaneous Diseases and continued in the laboratories of Pathology of the University of Pennsylvania, with the cooperation of Dr. Allen J. Smith, Dr. Charles K. Mills, and others, as a part of a series of investigations bearing upon the microparasitology and clinical aspects of poliomyelitis.



It is probable that the micrococci recently described and regarded as the specific etiologic agent of acute anterior poliomyelitis were met with early in the course of investigations bearing upon the etiology of this disease. Bülow-Hansen and Harbitz (5) and Harbitz and Scheel (6, 7) found a diplococcus in the cerebrospinal fluid of several cases of acute anterior poliomyelitis and referred to the work of Geirsvold (8), who found a diplococcus in the cerebrospinal fluid of twelve cases and claimed to have produced paralysis and death in experimental animals with them. Pasteur, Foulerton, and Maccormac (9) reported the discovery of a micrococcus in the cerebrospinal fluid of a case during life, which produced in rabbits symptoms resembling the disease in human subjects. Leiner and von Wiesner (10) and Krause and Meinicke (11) reported that these micrococci were not the etiologic agents of acute poliomyelitis, as had Flexner and Lewis, who first showed that the etiologic agent was filterable through dense filters and probably belonged to the filterable viruses (12); simultaneous and similar results were observed and reported by Landsteiner and Levaditi (13). Dixon, Fox, and Rucker (14) also found a diplococcus in the cerebrospinal fluid, nose, and throat of patients with acute anterior poliomyelitis, and Rucker made one of the fullest studies of this diplococcus apparently identical with that recently described by Mathers (2) and Nuzum and Herzog (4). While the latter have reported the successful infection of various laboratory animals with these cocci and the reproduction of a disease with clinical symptoms and lesions similar to those of acute poliomyelitis, the experiments of the former were negative throughout; an inoculated monkey succumbed with a hemorrhagic meningitis, but without clinical or histological evidences of anterior poliomyelitis.

Rosenow (3) has recovered what he calls a "peculiar polymorphous streptococcus" from the tonsils, brain, cord, mesenteric lymph glands, and once from the blood, but never from the cerebrospinal fluid of cases of acute poliomyelitis, which produced lesions and symptoms among the lower animals regarded as those of acute poliomyelitis. He has described changes in size and staining reaction of these streptococci according to the culture medium employed, the age of the culture, and whether they have been grown aerobically or anaerobically. The cocci were said to become very small under anaerobic conditions and to approach in size the globoid bodies described by Flexner and Noguchi; the small forms were found to be filterable through Berkefeld filters while the larger forms were not.

We have found various microorganisms and especially a diplococcus and a streptococcus not only in the cerebrospinal fluid but also in the tissues of the central nervous system and in various other internal organs of fatal cases; in no instance, however, have we been able to produce anterior poliomyelitis in monkeys and rabbits with any culture by intracranial, intravenous, or intraperitoneal injection of these microorganisms.

*Microorganisms Isolated.*

From the cerebrospinal fluid of poliomyelitic patients during life and from various tissues after death, we have cultivated anaerobically four different varieties of microorganisms as follows: (a) streptococci, (b) diplococci, (c) diphtheroids, and (d) Gram-negative bacilli.

As stated above, we have divided the micrococci into two groups; namely, those which grow out into long chains and present the characteristics of streptococci and those which assume a diplococcus arrangement in small clumps and short chains. Apparently Rose now believes that these are the same microorganism with a polymorphous nature. Before summarizing the results of our anaerobic cultures of the cerebrospinal fluid, blood, and tissues of poliomyelitic patients, the different microorganisms which we have recovered may be briefly described.

*Streptococcus.*

1. In cultures of tissues containing streptococci in an ascites-broth-kidney medium under sterile paraffin oil, macroscopic growth was first apparent after 5 to 9 days' incubation at 37°C.

2. Primary anaerobic and aerobic cultures and particularly subcultures of the streptococci grew in the form of a granular sediment at the bottom and sides of the tubes with a clear or but slightly cloudy supernatant medium. The diplococci, however, grew more diffusely.

3. Cultures under paraffin oil and in a Novy jar in an atmosphere of hydrogen grew somewhat more slowly than cultures under oil only.

4. Aerobic transplants from the anaerobic cultures to suitable solid or fluid media presented a visible growth within 48 hours.

5. Aerobic cultures of the streptococcus were readily secured by culturing emulsions of tissue in ascitic broth at 37°C. Anaerobic cultures required more time (at least 5 to 9 days, as stated above).

6. Films of 5 to 9 day anaerobic cultures showed short chains of Gram-positive cocci; longer chains were found later, although they may be found in the initial growth. As a rule, these cocci were round or slightly flattened; in a few instances the cocci in young anaerobic cultures have been elongated, while in older cultures the spherical shape was noted.

7. On defibrinated horse blood agar the colonies were small and of three varieties: (1) those which were non-adherent and produced a greenish pigment; (2) those which were non-adherent and hemolytic; and (3) those which were non-adherent and non-hemolytic.

8. Definite capsules were never found with any culture even after passage through mice, although films of cultures in ascitic broth stained after the method of Gram not infrequently showed slight unstained halos about the cocci suggestive of capsules.

9. In older anaerobic cultures the streptococci became smaller; after 14 days' incubation at 37°C. the majority of cocci were much smaller than those in fresh anaerobic and aerobic cultures. Large and small forms have been found together and the small cocci are easily decolorized during staining after the method of Gram.

10. Of sixteen cultures studied in relation to acid production with various carbohydrates, two were found to produce acid with inulin.

11. Aerobic and anaerobic cultures of streptococci were obtained from the cerebrum, cerebellum, pons, and cord after remaining in a 50 per cent mixture of pure neutral glycerol and sterile salt solution for periods of time varying from 10 days to 8 weeks.

The notable features of these streptococci were their slow multiplication in anaerobic cultures; the more rigid the anaerobiosis, the slower were the growths. The cocci became progressively smaller under anaerobic conditions, more easily decolorized with alcohol in the Gram method of staining, and more indefinite in outline as viewed microscopically.

Compared with films of mass cultures of the microparasite described by Flexner and Noguchi stained after the method of Gram but without counterstaining, our cocci were somewhat larger. The organism of Flexner and Noguchi occurs in short chains and irregular clumps, and while in our older anaerobic cultures we could find occasional cocci comparable with them in their minute size, yet the majority of our preparations showed uniformly distinctly larger forms.

### *Diplococci.*

The most frequently cultivated microorganism in the cerebrospinal fluid and tissues of acute anterior poliomyelitis and apparently that first described occurs in the form of a Gram-positive diplococcus arranged in isolated pairs, tetrads, short chains, and irregular clumps. This diplococcus seems neither to have been named nor has its relation to known diplococci been established. The important morphological and biological characters of our cultures of these diplococci correspond closely with those described by various investigators.

1. In anaerobic cultures in ascites-broth-kidney medium of cerebrospinal fluid and emulsions of tissue containing this microorganism growth first appeared after 3 days' incubation at 37°C. Aerobic cultures presented macroscopic growth in 24 hours. In fluid medium the growths were diffuse with the gradual formation of a slimy sediment along the sides and bottom of the tubes.

2. Subcultures of the anaerobic cultures on ordinary culture media grew luxuriantly, producing whitish moist colonies resembling *Staphylococcus albus*.

3. Films of the aerobic and young anaerobic cultures showed a Gram-positive coccus usually arranged in diplococcus formation in chains of four or five pairs and in small clumps. Films of older anaerobic cultures (14 days or more) showed that the majority of these cocci had become smaller and many were easily decolorized by alcohol in the Gram stain.

4. Aerobic subcultures usually showed a staphylococcus grouping with a loss of the diplococcus formation. On solid media the tendency for the formation of short chains was lost.

As with the streptococci these diplococci tend to become smaller under strict anaerobic conditions as do cultures of *Staphylococcus albus* under similar conditions. These diplococci have many properties in common with varieties of diplococci found in the skin by one of us (15), and except for the reason that they have been found in tissues and cerebrospinal fluid collected under apparently aseptic conditions, we should be strongly inclined to regard them as microorganisms derived from the skin and mucous membranes.

### *Diphtheroid Bacilli.*

These bacilli, which were short, solid, or occasionally granular in appearance and Gram-positive, did not present any difficulties in their recognition. They grew slowly under anaerobic conditions, but luxuriantly on appropriate media under aerobic conditions, producing large whitish colonies resembling the pseudo-diphtheria bacillus rather than the true virulent bacillus. They were non-virulent for animals and their biological properties were those commonly found with cultures of diphtheroids recovered from enlarged lymphatic glands and mucous membranes.

### *Gram-Negative Bacilli.*

In anaerobic and aerobic cultures of emulsions of poliomyelitic tissues and also of cerebrospinal fluid during life, two varieties of Gram-negative bacilli were found. (1) The usual variety was a short bacillus which morphologically and biologically appeared to be a member of the *B. coli* group. The majority produced gas with dextrose, produced indole, and coagulated milk. (2) In a few cultures

of the tissues a second Gram-negative bacillus was found which was much smaller than the preceding and resembled the influenza bacillus. Further statements regarding these bacilli cannot be made at this time except to state that we do not regard them as *B. influenza*.

*Results of Anaerobic Cultures of Cerebrospinal Fluid in Acute Anterior Poliomyelitis.*

Anaerobic cultures were made of the cerebrospinal fluid from 106 cases of acute anterior poliomyelitis by placing 1 or 2 cc. of the freshly drawn fluid collected under aseptic precautions in tubes of ascitic fluid or ascitic fluid and broth containing sterile rabbit kidney and covered with sterile paraffin oil. After incubation for 5 days or more the cultures were examined and studied; in Table I is given a summary of the results of these cultures, showing the different bacteria found. Not infrequently a culture of one fluid contained two or even three different microorganisms.

The majority of these cultures were made within a few hours after the admission of the patients to the Philadelphia Hospital for Contagious Diseases and at varying intervals after the onset of symptoms.

TABLE I.

*Anaerobic Cultures of the Cerebrospinal Fluid of 106 Cases of Acute Anterior Poliomyelitis.*

Microorganism.	Cultures showing presence of microorganism.
Sterile.....	16
Streptococci.....	None.
Diplococci.....	48
Gram-negative bacilli.....	22
Diphtheroids.....	22
<i>B. subtilis</i> .....	20

As shown in this table, definite streptococci were not found; the diplococcus was found in forty-eight fluids, or 45 per cent of those cultured. As previously stated, this microorganism may occur in pairs or short chains in the anaerobic cultures, but aerobically and on solid medium a staphylococcus arrangement is usual.



Gram-negative bacilli and diphtheroids were found in 20 per cent of cultures. *Bacillus subtilis* was also found in 19 per cent. This microorganism is of course a contamination, and not only indicates the difficulties of avoiding contamination in the collection of spinal fluids from a large number of small, sick children, but casts doubt on the series as a whole (16).<sup>1</sup>

*Results of Anaerobic Cultures of Various Tissues in Acute Anterior Poliomyelitis.*

Anaerobic cultures of various tissues from fatal cases of poliomyelitis were made during or immediately after autopsy, in ascites-kidney and ascites-broth-kidney media. All the cultures reported herein were cultivated under paraffin oil only and not in the Novy jar. The tissues removed at autopsy were collected with care to exclude bacterial contamination as much as possible, but the conditions were not such as always to accomplish this end. Hence the portion of tissue selected for culture was dipped momentarily in boiling salt solution or water before being cultured, obviously an imperfect method of securing even superficial sterilization of the tissues. Here again, as Table II shows, the variety of bacterial forms cultivated indicates that either postmortem or agonal invasion had taken place or unavoidable contamination had occurred during removal of the organs.

As shown in Table II, streptococci were found in the brain and cord of at least 50 per cent of our cases. The presence of streptococci in various other internal organs including the thymus gland, liver, spleen, mesenteric glands, kidneys, suprarenal glands, and pancreas was also noteworthy. While a streptococcus has been reported in the lymphatic glands, yet its widespread distribution in the tissues of poliomyelitic patients has not been determined heretofore, as indicated in our studies.

<sup>1</sup> The experience of the Department of Health of the City of New York with cultures of the cerebrospinal fluid is valuable in this connection (16). One difference in the results may be attributable to the fact that we employed the anaerobic method of cultivation which develops growth under conditions in which the aerobic cultures do not.



TABLE II.

*Results of Anaerobic Cultures of Various Tissues in Fatal Cases of Anterior Poliomyelitis.*

Organ.	Total No. examined.	Results of cultures.				
		Sterile.	Streptococci.	Diplococci (staphylococci).	Diphtheroids.	Gram-negative bacilli.
Cerebrum.....	8	2	4	3	0	3
Cerebellum.....	8	1	4	2	0	2
Pons and medulla.....	7	2	6	4	0	2
Cord.....	8	1	4	4	1	3
Exudate on cord.....	6	0	5	2	2	1
Thymus gland.....	2	0	2	0	0	0
Lungs.....	3	0	3	2	0	0
Liver.....	3	1	1	2	0	0
Spleen.....	3	1	3	1	0	0
Kidneys.....	3	0	2	1	1	0
Mesenteric glands.....	4	0	3	3	0	1
Pancreas.....	4	2	1	1	0	0
Suprarenals.....	2	1	1	0	0	0
Tonsils.....	2	0	2	2	1	0

The streptococci from the internal organs resembled those from the brain and cord, except that the former showed a tendency to grow in longer chains.

The diplococci were likewise found not only in the brain and cord but also in various internal organs.

The diphtheroids were found infrequently, while Gram-negative bacilli were found mostly in the cultures of the brain and cord.

*Anaerobic Cultures of the Blood in Acute Anterior Poliomyelitis.*

Blood cultures were made of twenty cases of anterior poliomyelitis during the acute stages of the disease by withdrawing 1 to 3 cc. of blood from a vein at the elbow under aseptic precautions and culturing in ascites-broth-kidney medium under paraffin oil. A streptococcus was recovered in one instance, while a staphylococcus was secured in ten. The latter in the fluid medium had a diplococcus arrangement while transplants on solid medium such as plain or glucose agar yielded profuse whitish colonies resembling *Staphylococcus*

*albus*. These cocci were practically indistinguishable from the micrococci cultivated from the spinal fluid. Since the blood has been cultured repeatedly without result, the question arises again whether contamination was not also the explanation of the presence of diplococci in the blood.

### *Filtration Experiments.<sup>2</sup>*

Since Flexner and Lewis demonstrated that the etiologic agent of acute anterior poliomyelitis is filterable through filters which hold back such microorganisms as *Micrococcus prodigiosus*, filtration experiments are of considerable interest and importance in the study of the etiology of this disease. The later experiments of Flexner and Noguchi showed that the minute globoid bodies were filterable, and Rosenow has reported that the small forms of the polymorphous streptococcus are filterable through Berkefeld N filters.

We have conducted a number of experiments with salt solution emulsions of tissues containing diplococci and streptococci and with various pure cultures of these and other microorganisms. In the absence of reliable Berkefeld filters we have employed the small Kitasato (fine) and larger Pasteur-Chamberland (fine) candles. The tissues had been in 50 per cent glycerol and salt solution for varying intervals of time, but cultures of the emulsions made at the time of the filtrations showed the presence of viable microorganisms.

Pieces of tissue about the size of a bean were washed several times in sterile water and ground in sterile mortars with sterile sand, with the addition of about 15 cc. of sterile salt solution. The emulsion was then centrifuged or filtered through sterile paper and anaerobic cultures were prepared. The emulsions were then passed through the various sterilized filters with the aid of a suction pump and anaerobic cultures made of the first 3 cc. of filtrate and of larger amounts of filtrate.

Pure cultures of various microorganisms were first subcultured anaerobically and then passed through the candles and cultured in the same manner.

The results of these experiments are summarized in Tables III and IV.

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<sup>2</sup> We are indebted to Dr. Bertha M. Meine for assistance in conducting a number of these experiments.

TABLE III.

*Results of Filtration Experiments with Emulsions of Poliomyelitic Tissues.*

Material (glycerolated tissues).	Microorganisms present.	Filter used.	Amount of filtrate cultured.*	Results of anaerobic cultures of filtrates.†
Emulsion of cord (M).	Diplococci, streptococci.	Kitasato.	First 3 cc.	Sterile.
" " " (K).	Diplococci, streptococci, Gram-negative bacilli.	"	" 3 "	"
" " " (K).	Diplococci, streptococci, Gram-negative bacilli.	"	3 cc. of 15 cc. of filtrate.	Diplococci, streptococci, Gram-negative bacilli.
" " " (H).	Diplococci.	Pasteur-Chamberland.	First 3 cc.	Sterile.
" " " (C).	Streptococci, diplococci.	Pasteur-Chamberland.	" 3 "	"
" " " (C).	Streptococci, diplococci.	Pasteur-Chamberland.	3 cc. of 12 cc. of filtrate.	"
" "cerebrum(M).	Diplococci.	Kitasato.	First 3 cc.	"
" " " (H).	"	"	" 3 "	"
" " " (H).	"	Pasteur-Chamberland.	" 3 "	"
" "pons(M).	Streptococci (pure culture).	Kitasato.	" 3 "	"
" " " (M).	Streptococci (pure culture).	Pasteur-Chamberland.	" 3 "	"
" " " (K).	Streptococci, diplococci.	Kitasato.	3 cc. of 16 cc. of filtrate.	Growth of streptococci and diplococci.
" " " (K).	Streptococci, diplococci.	Pasteur-Chamberland.	3 cc. of 16 cc. of filtrate.	Sterile.

\* Cultures in ascites-broth-kidney medium under paraffin oil.

† After 28 days' incubation at 37°C.

As shown in Table III, the filtrate of the emulsions of tissues with both the small Kitasato and larger Pasteur-Chamberland filters was invariably sterile if the first 3 cc. or less of filtrate were cultured; when larger amounts of emulsion were filtered and cultured, growths

TABLE IV.

*Results of Filtration Experiments with Pure Cultures of Microorganisms from Poliomyelitic Tissues.*

Culture* (ascites-broth-kidney medium).	Microorganisms present.	Filter used.	Amount of fil- trate cultured.†	Results of anaerobic cultures of filtrates.‡
5 day anaerobic.	Streptococci (large forms).	Kitasato.	First 3 cc.	Sterile.
5 " "	Streptococci (large forms).	"	3 cc. of 10 cc. of filtrate.	"
7 " "	Streptococci (mostly large forms).	Pasteur-Chamberland.	First 5 cc.	"
14 " "	Streptococci (many small forms present).	Kitasato.	" 3 "	"
14 " "	Streptococci (many small forms present).	"	3 cc. of 17 cc. of filtrate.	Streptococci.
17 " "	Streptococci (many small forms present).	Pasteur-Chamberland.	First 4 cc.	Sterile.
17 " "	Streptococci (many small forms present).	Pasteur-Chamberland.	4 cc. of 19 cc. of filtrate.	"
19 " "	Streptococci (many small forms present).	Pasteur-Chamberland.	5 cc. of 31 cc. of filtrate.	Streptococci.
8 " "	Diplococci.	Kitasato.	First 3 cc.	Sterile.
8 " "	"	"	3 cc. of 12 cc. of filtrate.	"
8 " "	Gram-negative bacilli.	"	First 3 cc.	"
8 " "	" "	"	3 cc. of 11 cc. of filtrate.	"

\* Subcultures of all of these in ascites-broth-kidney medium preliminary to filtration showed the presence of viable microorganisms.

† In ascites-broth-kidney medium under paraffin oil.

‡ After incubation for 11 to 28 days at 37°C.

were sometimes secured with the Kitasato filtrates. The amount of filtrate used in these experiments is therefore of considerable importance.

Experiments with pure cultures of various microorganisms showed

in general that larger amounts of filtrate may contain microorganisms, while the first few cubic centimeters may be sterile; also that streptococci in older anaerobic cultures (containing many small forms) may be filterable, while younger cultures, containing mostly larger forms, are not. There is no doubt that the streptococci and also the diplococci secured by us from poliomyelitic tissues tend to become much smaller when grown in fluid medium under paraffin oil, and as shown in the above experiments these small forms may be filterable under conditions which hold back larger forms. Of even more importance, however, is the amount of culture passed through a candle, as the passage of large amounts may wash through a number of the microorganisms.

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*Results of Animal Inoculation Tests.*

We have injected cultures of the various microorganisms into rabbits, and a few cultures of streptococci and diplococci into monkeys intracranially, intravenously, and intraperitoneally. All the animals were carefully observed for clinical evidences of poliomyelitis and after death sections were prepared of the brain and cord and examined for histological evidences.

*Rabbit Inoculation Experiments.*—The cultures injected into rabbits were derived from the following sources.

No. of cultures.	Cultures.	Source.
14	Streptococci.	Poliomyelitic cords.
5	"	" brains.
2	"	Spleen.
3	"	Mesenteric glands.
5	Diplococci.	Cerebrospinal fluids.
3	"	Poliomyelitic cords.
2	"	" brains.
4	Diphtheroids.	" tissues.
3	"	Cerebrospinal fluids.
6	Gram-negative bacilli.*	Poliomyelitic tissues.

\* These bacilli were not of the colon group.

With the exception of two cultures all were transplants from the original anaerobic ascites-broth-kidney cultures, in order to eliminate



to a large extent the possible injection of original tissue present in the primary culture and to insure the injection of pure cultures. The aerobic cultures used for injection had been incubated at 37°C. for 24 hours, and in all instances stained films showed the presence of numerous microorganisms. The doses administered are given in Table V. The animals varied in weight from 1,400 to 1,600 gm.

TABLE V.

*Results of Rabbit Inoculation Tests.*

Microorganism.	No. of cultures injected.	Route of inoculation.	Dose.	Results.
Streptococci.	18	Intracranial.	cc. 0.5-0.8	No paralyses; six deaths 4, 18, 20, 21, 26, and 26 days later.
"	24	Intravenous.	1.0*	No paralyses; one arthritis; seven deaths 1, 2, 9, 15, 19, 35, and 56 days later.
"	24	Intraperitoneal.	2.0*	No paralyses; three deaths 3, 7, and 10 days later.
Diplococci.	7	Intracranial.	0.5-0.6	No paralyses; one death 28 days later.
"	10	Intravenous.	1.0	No paralyses; one death 3 days later.
"	10	Intraperitoneal.	2.0	No paralyses; no deaths.
Diphtheroids.	4	Intracranial.	0.5-0.6	No paralyses; one death 36 days later.
"	7	Intravenous.	1.0	No paralyses; one death 34 days later.
"	7	Intraperitoneal.	2.0	No paralyses; one death 36 days later.
Gram-negative bacilli.	3	Intracranial.	0.5-0.6	No paralyses; three deaths 11, 14, and 21 days later.
" "	6	Intravenous.	1.0	No paralyses; three deaths 1, 25, and 35 days later.
" "	6	Intraperitoneal.	2.0	No paralyses; one death 28 days later.

\* Dose per kilo of body weight.

As shown in Table V, which summarizes the number of different cultures of various microorganisms injected in different routes, true clinical and histological evidence of poliomyelitis was not observed in a single instance.

The streptococci produced arthritis in one rabbit and the intracranial injections were followed in several animals by a slight meningitis with recovery of streptococci at autopsy.

The streptococci caused more deaths than the diplococci, diphtheroids, and Gram-negative bacilli.

The intravenous injections were followed in several instances by the development of fatal pleuritis and pericarditis, from which lesions and from the blood of the heart, streptococci were recovered. On the other hand, the deaths of many of these animals and particularly those succumbing 3 and 4 weeks or longer after injection were due to secondary and unrelated causes.

In several of the animals injected with the diphtheroids and Gram-negative bacilli and succumbing 2 weeks or more later, these microorganisms were not recovered, whereas cultures of the heart and various internal organs frequently showed the presence of streptococci, indicating that the animals are subject to independent and secondary fatal infections with streptococci.

*Monkey Inoculation Experiments.*—The inoculation experiments have been limited to four animals. The intracerebral injection of the recently cultivated diplococci failed to produce anterior poliomyelitis (No. 1); likewise the intracerebral injection of five different strains of streptococci failed, producing, however, in one case a meningitis (No. 2); likewise the intravenous (No. 3) and intraperitoneal (No. 4) injections of streptococci failed to produce symptoms or lesions of poliomyelitis. The brief protocols of these four experiments are as follows:

*Monkey 1.*—*Macacus rhesus*; inoculated intracerebrally under ether anesthesia with 0.8 cc. of a mixture of the first anaerobic ascitic broth subcultures of recently glycerolated cords of four cases of poliomyelitis. The cultures were incubated for 4 days and showed numerous diplococci arranged in pairs and short chains. The animal promptly recovered from the anesthetic and operation, showed a slight disinclination to move about for 3 days, but has never showed any weakness or paralysis (44 days' observation). Subcultures were employed to aid in the elim-

ination of the injection of original tissue; the diplococci were, therefore, 8 days in artificial medium before inoculation.

*Monkey 2.*—*Macacus rhesus*; inoculated intracerebrally under ether anesthesia with 0.8 cc. of a mixture of 3 day aerobic ascitic broth cultures of five different strains of streptococci recovered from two cords, one cerebrum, one spleen, and one thymus gland of five different cases of poliomyelitis. Two strains were in the first transfer, and the remaining three were in the third transfer from the original anaerobic cultures. The animal promptly recovered from the anesthetic and operation and developed a severe meningitis lasting over 2 weeks, with pus and streptococci in the cerebrospinal fluid. The animal did not move about or use its hind legs, but repeated tests failed to discover paralysis.

*Monkey 3.*—*Cebus capucinus*; inoculated intravenously with 5 cc. of a mixture of 4 day aerobic ascitic broth cultures of five different strains of streptococci in the second and third transfers from original anaerobic ascites-kidney-broth cultures of three cords, one pons, and one spleen of five different cases of poliomyelitis. This animal presented a mild rise in temperature over a period of 2 days following the injection, with no other disturbances and no weakness or paralysis (56 days' observations).

*Monkey 4.*—*Cebus capucinus*; inoculated intraperitoneally with 5 cc. of the same mixture of five different strains of streptococci given to Monkey 3. This animal has not shown weakness or paralysis (56 days' observations).

In considering this series of experiments on monkeys note should be taken of the fact that they reproduce chiefly the method of inoculation which succeeds with the filtered poliomyelitic virus. In one instance only was an intravenous injection given and in it the dose was below that employed by Rosenow. However, the large quantity of cultures inoculated should have sufficed to develop symptoms of poliomyelitis had the streptococci used possessed the power of inducing that disease.

#### DISCUSSION AND SUMMARY.

Four different varieties of easily cultivated microorganisms have been cultured from the cerebrospinal fluid and tissues of cases of acute anterior poliomyelitis; namely, a streptococcus, a diplococcus, diphtheroids, and Gram-negative bacilli. It is not contended that they were all inherent in the tissues; a part were doubtless extraneous.

The streptococci and diplococci may be considered as the most significant of the bacteria cultivated and are distinguishable from each other by biological tests.

The streptococci grew both aerobically and anaerobically; under anaerobic conditions growth was slow, the cocci became small and round, and were more easily decolorized with alcohol in the Gram stain. They were not found in the anaerobic cultures of 106 cerebrospinal fluids; they were found in one of twenty anaerobic blood cultures and frequently in the cerebrum, cerebellum, pons and medulla, cord, tonsils, lungs, liver, kidneys, spleen, pancreas, thymus gland, suprarenal glands, and mesenteric glands of fatal cases.

The diplococci are Gram-positive and, transplanted to solid media, yield luxuriant growths and a staphylococcus grouping. They grew aerobically and anaerobically, but more slowly under the latter condition, and the cocci became smaller and more rounded. Diplococci were found in the anaerobic cultures of 48 of 106 cerebrospinal fluids; also in the cerebrum, cerebellum, pons and medulla, cord, tonsils, lungs, liver, kidneys, spleen, pancreas, and mesenteric glands of fatal cases.

The filtrates of emulsions of tissues containing streptococci and diplococci passed through fine Kitasato and Pasteur-Chamberland filters were sterile unless large amounts of filtrates were collected. The amount of filtrate collected and cultured is therefore of considerable importance in filtration experiments.

The small forms of streptococci and diplococci in old anaerobic cultures are filterable with these filters, while young aerobic cultures containing large forms are not, unless large amounts of culture are filtered.

Intracranial, intravenous, and intraperitoneal injection of these easily cultivated streptococci, diplococci, diphtheroids, and Gram-negative bacilli failed to produce paralysis in rabbits or monkeys. With two exceptions all the cultures were transplants from the original anaerobic ascites-broth-kidney cultures of cerebrospinal fluid and various tissues. Arthritis and meningitis were produced by the streptococci, but there were neither clinical nor histological evidences of true poliomyelitis.

Occasional bacteriological studies since 1898 have shown that easily cultivated micrococci and bacilli may be present in the cerebrospinal fluid and tissues of the central nervous system of persons suffering with acute anterior poliomyelitis. The majority of bacteriol-

ogists have found the cerebrospinal fluid, blood, and nervous organs sterile. Opinions have varied in regard to the significance of the organisms and the micrococci in particular, but the consensus of opinion has been to the effect that they are secondary invaders and unable of themselves to produce poliomyelitis in the lower animals. After allowing for contaminations due to technical errors in securing specimens, the total number of observations indicates that easily cultivated micrococci occur sometimes in the brain and cord of persons suffering from epidemic poliomyelitis. Our studies have shown that they may be found not only in these locations, but also in the spleen, kidneys, suprarenal glands, and other organs. It is not known that they exert an influence in this disease, although they may possibly give rise to the production of antibodies, assuming their entrance not to be wholly agonal, as the cultures of streptococci are frequently of sufficient virulence to produce meningitis in rabbits and monkeys. Our experiments are in accord with those of other investigators who found that these microorganisms do not produce poliomyelitis in the lower animals, and are therefore in sharp contrast with the recent reports which would attribute an etiologic relationship of streptococci and allied organisms to that disease. At present this wide divergence of result cannot be accounted for, but it does not seem that it is possible for it to reside in any condition of the cultures employed by us as they were obtained from undoubted cases of epidemic poliomyelitis and inoculated in early generations.

As regards these easily cultivatable microorganisms, we agree at present with those who regard them as secondary and probably terminal invaders rather than the actual etiologic agent of the disease.

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## EXPLANATION OF PLATE 66.

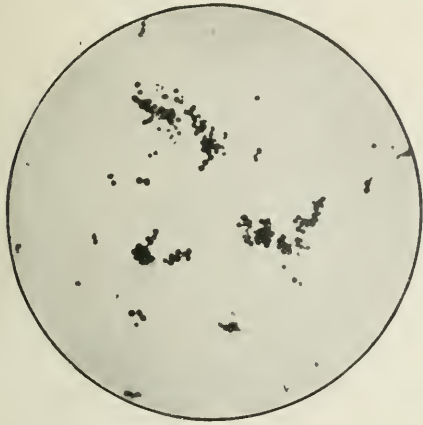
FIG. 1. Film of a 2 day aerobic subculture of diplococcus in ascites-broth-kidney medium; from an anaerobic culture of poliomyelitic cord in the same medium. Shows large forms.  $\times 1,000$ .

FIG. 2. Film of an 18 day anaerobic culture of diplococcus in ascites-broth-kidney medium; same culture as shown in Fig. 1. The cocci have become smaller in size and more easily decolorized by alcohol in the Gram stain.  $\times 1,000$ .

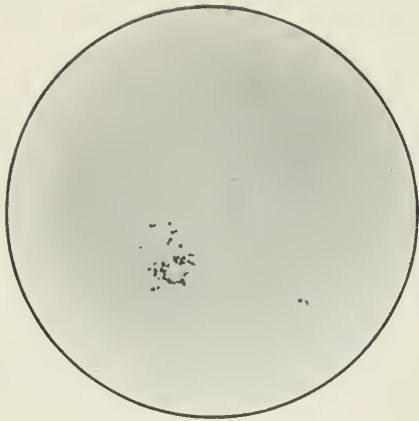
FIG. 3. Film of a 5 day anaerobic culture of streptococcus in ascites-broth-kidney medium; from the pons of a fatal case of acute anterior poliomyelitis.  $\times 1,000$ .

FIG. 4. Film of an 11 day anaerobic culture of streptococcus in ascites-broth-kidney medium; same culture as shown in Fig. 3; some smaller forms of cocci tending to become Gram-negative are seen.  $\times 1,000$ .

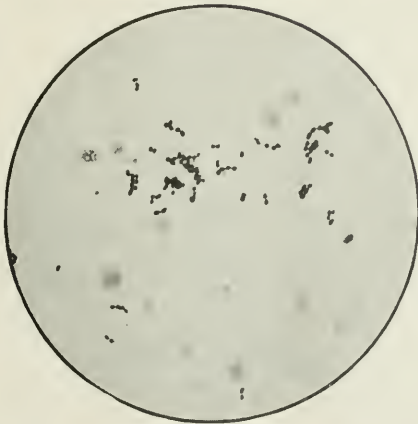
FIG. 5. Film of a 3 day aerobic culture of streptococcus in ascites-broth-kidney medium; from the thymus gland of a fatal case of acute anterior poliomyelitis.  $\times 1,000$ .



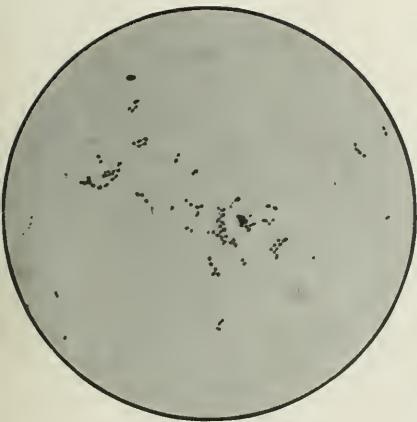
1



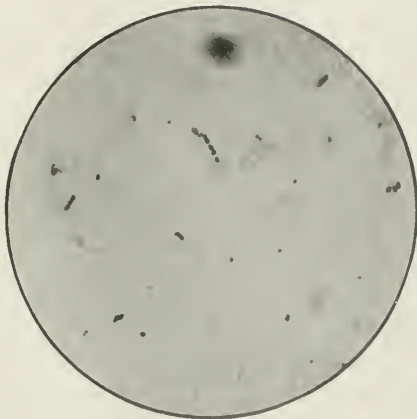
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5



# HENLE'S REACTION OF THE CHROMAFFIN CELLS IN THE ADRENALS, AND THE MICROSCOPIC TEST FOR ADRENALIN.

BY TOMOSABURO OGATA AND AKIRA OGATA.

*(From the Pathological Institute, and the Chemical Laboratory of the Pharmaceutical Institute of the Imperial University of Tokyo, Tokyo, Japan.)*

PLATE 67.

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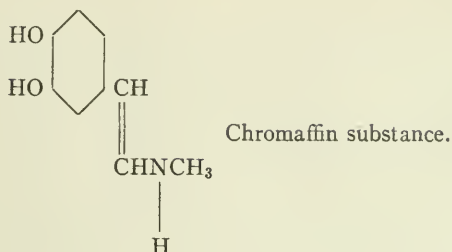
## HISTORICAL.

In 1865 Henle (1) discovered a brown coloration of the medullary cells of the adrenals when remaining in a solution of potassium dichromate, which at that time was generally used in histology for the hardening of tissues. Henle's reaction has since been confirmed by many investigators, and was also found to occur in other parts of the body; e.g., in the carotid gland, in the sympathetic ganglia, in Zuckerkandl's gland, etc. The cells with a positive reaction are always found in groups, mixed with sympathetic ganglion cells; and numerous embryological investigations have made it clear that both are derived from the same ectodermal mother cells. On the other hand, comparative anatomical studies have shown that the cortex (interrenal system) and the medulla (suprarenal system) of the adrenals are genetically foreign to each other, and in lower vertebrates exist as two independent organic systems. On the basis of these results it can scarcely be doubted that the medullary portion of the adrenals represents, with all tissues giving a positive chrome reaction, a system which stands in close relation to the sympathetic nervous system (Kohn (2), Biedl (3), Wiesel (4, 5)). This characteristic behavior towards chromates was taken into consideration in naming these cells; e.g., chromaffin (Kohn (2)), chromophil (Stilling (6)), chrome brown, or pheochrome (Poll (7)). The term chromaffin is the most commonly used. This term, as Kohn has stated, expresses only the ability of the cells, tissue, or system to be colored brown by dichromate or chromic acid, when in a fresh state, while with the chromaffin substance of Borberg and Ciaccio (17, 18) the term chromaffin indicates chemical affinity. While the chrome reaction was thus established as a characteristic of the chromaffin cells, numerous chemico-physiological investigations convinced us, on the other hand, that these cells produce adrenalin and supply it to the vascular system. This substance is considered an im-

portant hormone for the regulation of the vascular tonus. Langlois and Vincent were the first to show that adrenal extract causes an increase of blood pressure. Later Takamine isolated from adrenal extract a chemical compound which increased blood pressure, and which he named adrenalin. According to some authors (Biedl, Wiesel, etc.), the extract of extracapsular chromaffin tissues, *e.g.*, those of Zuckerkandl's gland, shows the same property of raising blood pressure as that of the adrenals. It is thus clear that adrenalin is a constant chemical constituent of chromaffin tissues. Biedl proposed the terms adrenal cells, adrenal tissue, adrenal bodies, adrenal organs, and adrenal system. He did not, however, always use the term adrenal cells, but substituted for it that of chromaffin cells (Kohn). In France the terms "*cellules adrénalogènes*" (Bonnamour (8)) and "*cellules adrénalinogènes*" have since been in use.

As has been explained above, two constant characteristics, affinity for chromic acid and chromates (Henle's reaction) as a histological, and the production of adrenalin as a physiological characteristic, were established in certain kinds of cells. However, it has not yet been made entirely clear whether these two peculiarities exist accidentally or whether there is a distinct relation between them. While the experiments of Kahn (10), Schur and Wiesel (11), etc., showed parallelism between the degree of the reaction and the adrenalin content, contrary results were published by Ingier and Schmorl (12), Popielski (13), and others. Biedl states that Henle's chrome reaction can serve only as a preliminary orientation as to the quantity of adrenalin contained. Though the presence of a definite relation between the two phenomena is assumed, proof of it is lacking. On the other hand, some investigators, as Mulon (14), Elliott and Tuckett (16), Ciaccio (17), Borberg (18), and others, believe that the chrome reaction is a purely chemical process, which occurs between chromates (chromic acid) and adrenalin or its derivatives. Borberg described this process in detail. He stated that adrenalin solution after the addition of a solution of potassium dichromate gradually becomes more and more reddish brown until in the course of a few hours a slowly increasing dark brown precipitate appears. According to him, the precipitate showed, besides its similarity in color, in its behavior towards various solvents complete agreement with the chrome reaction in the adrenal medulla. He therefore identified both reactions in the test-tube and on the slide. The reaction in the test-tube he explained thus: that potassium dichromate or the chromic acid resulting from the interaction of this salt and the acetic acid in the solution forms an insoluble brownish chemical compound. But he believed that the reaction on the slide was caused not by adrenalin itself but by substances in the preliminary stage of adrenalin formation (adrenalinogen, pre-adrenalin), which would be oxidized into adrenalin derivatives combined with chromates. This so called chromaffin substance may have, according to his hypothesis, the following chemical structure.





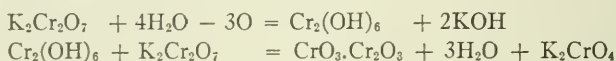
The relation between the chrome reaction and the presence of adrenalin is, however, not yet proved. This is why Biedl maintains that the chrome reaction could only serve as a preliminary orientation as to the quantity of adrenalin contained.

#### EXPERIMENTAL.

We believe that we have succeeded in solving this question. Our materials were mostly the fresh adrenals of healthy domestic animals, as cattle, horses, pigs, sheep, dogs, rabbits, guinea pigs, and rats. Human adrenals were also used, but were found not to be so well suited for our purpose as in them the reaction was much weaker than in the fresh adrenals of healthy animals. We also investigated in the test-tube the chemical process between adrenalin and potassium chromate, for the reaction can here be easily studied. It was only later we learned that Borberg had also investigated the reaction in this manner. But his results were different from ours. We too observed that an adrenalin solution turns brown on the addition of a solution of potassium chromate, to be followed by a dark brown precipitate which slowly increases. At first we believed with him that the precipitate was a chemical compound of chromic acid with some derivative of adrenalin, which arises by oxidation from adrenalin and dissolves in water, showing red coloration, for we perceived, in mixing both solutions, first the change of color into red and then the brownish precipitate. By closer examination of the properties of the brownish precipitate we learned that the latter was not a complicated organic compound but a simple inorganic one, namely chromium dioxide ( $\text{CrO}_2$ ).

There are various methods for producing chromium dioxide; for instance, by introducing nitric oxide into a solution of potassium chro-

mate (Schweitzer (19)), by adding a solution of potassium chromate to a solution of chromium sulfate (Maus (20)), by digesting chromic acid solution and chromium hydroxide (Beusch (21)), by adding a solution of sodium thiosulfate to one of potassium chromate (Popp (22)), or reducing potassium dichromate by sunlight in the presence of alcohol (Vogel (23)). The process may be explained thus: that chromates are first reduced to chromium dioxide and the latter combines with the existing chromic acid into chromium dioxide according to the following equation.



It is conceivable that adrenalin, which is easily oxidized and therefore can exert a reducing effect upon other compounds, *e.g.*, the reduction compounds mentioned above, extracts part of the oxygen from the potassium dichromate, thus forming chromium dioxide.

If in the experiment mentioned above the precipitate in the test-tube is examined, it is found to agree in appearance and other properties with chromium dioxide, which has been extracted according to one of the methods described. The precipitate is a brownish powder and appears under the microscope in the form of yellow-brown amorphous granules, is insoluble in various solvents, such as alcohol, ether, chloroform, xylol, benzene, etc., but is easily soluble in caustic potash and mineral acids. In acetic acid and formic acid it is not easily soluble, but if these organic acids are present in the original mixture its elimination is interfered with. When dried at 100°C. it becomes dark brown; when brought to a glow it gives off no carbon dioxide, proving the absence of organic compounds. Quick heating causes it to flare up, as Schweitzer has observed with chromium dioxide.

If this precipitate, which we believe to be chromium dioxide, is washed, the water appears yellow on account of the admixture of a surplus of potassium dichromate, and further washings make the color disappear to a yellowish trace which cannot be got rid of. The same fact has been observed with chromium dioxide. Schiff (24) washed 1 gm. of chromium dioxide for 10 hours daily for 6 weeks, but chromic acid was still seen to dissolve. Popp also mentions that if chromium dioxide is washed until color no longer appears, it changes

to a new compound,  $2\text{Cr}_2\text{O}_3 \cdot \text{CrO}_3$ . For explanation of this chromium dioxide might be regarded as a loose compound of chromium oxide and chromic acid, so that in washing, a part of the chromic acid is continually dissolved. When the brownish precipitate was allowed to remain for several days on the water bath, a greenish shade of color gradually appeared; and if dried at a gradually increasing temperature it became black and gradually decreased in weight. This agrees with the fact observed with chromium dioxide, that its hydroxide does not easily give off water, and only under high temperature changes into green chromium oxide, setting oxygen free.

In order to convince ourselves further that the precipitate is chromium dioxide, we made it glow and examined the residue (chromium oxide). The method was as follows. To a 1 per cent solution of adrenalin hydrochloride a solution of potassium dichromate was added in excess; it was left for 3 days at  $37^\circ\text{C}$ ., centrifuged, and poured off. To this, water was added and the same operation was repeated several times, until the upper layer retained the same yellowish color. Absolute alcohol was then added to the precipitate, the mixture was shaken for 30 minutes, again centrifuged, and treated with ether, after which a brown powder was obtained, which was dried at  $170^\circ\text{C}$ . in a platinum crucible until the weight remained constant. This powder was gradually heated to red heat and the green residue, chromium dioxide, was weighed.

0.2026 gm. substance gave 0.1648 gm. chromium oxide.

0.1648 gm. of chromium oxide gave 0.1822 gm. of  $2\text{CrO}_2$  or 0.2017 gm. of  $2\text{CrO}_2 + \text{H}_2\text{O}$ .

From this it follows that the substance must have the molecular formula  $2\text{CrO}_2 + \text{H}_2\text{O}$ .

The following formulas have been given for the hydrate of chromium dioxide, dried at various temperatures.

Observer.	Formula.	Temperature.
		$^\circ\text{C}$ .
Popp.	$2\text{Cr}_2\text{O}_3 \cdot \text{CrO}_3 + 9\text{H}_2\text{O}$	Not given.
Kopp.	$\text{CrO}_2 + 2\text{H}_2\text{O}$	Room.
Vogel.	$\text{CrO}_2 + \text{H}_2\text{O}$	100
Hinz.	$\text{CrO}_2 + \frac{1}{4}\text{H}_2\text{O}$	200
"	$\text{CrO}_2$	250

As the substance was dried at  $170^{\circ}\text{C}.$ , the formula  $2\text{CrO}_2 + \text{H}_2\text{O}$  ( $\text{CrO}_2 + \frac{1}{2}\text{H}_2\text{O}$ ) stands almost exactly between the third and fourth, and also agrees in this respect with the supposed hydrate of chromium dioxide.

As chromium dioxide is an amorphous substance, only a relative purity can be reached by washing. If the latter is continued too long, the chromium dioxide molecule loses part of its chromic acid; if it is not continued long enough, the precipitate may still contain some potassium chromate. It is therefore necessary to follow exactly the method of others in order to compare the results.

By the above investigation we believe we have established that the precipitate obtained by mixing solutions of adrenalin and dichromate in the test-tube is chromium dioxide.

We then prepared frozen sections of the adrenals, in which we had effected a chrome reaction. We left them in the solutions for a long time to test the conditions of solubility. In these sections we were able to ascertain the same conditions as those in the test-tubes. The shade of color in the sections was exactly that of chromium dioxide. By heating chromated adrenals to red heat we obtained a relatively large amount of chromium oxide. Though we were unable to analyze directly the brownish substance in the tissues, we may assume, according to the above investigation, that Henle's reaction is simply a reducing phenomenon of adrenalin upon dichromate of chromic acid. If the amount of adrenalin is great, a correspondingly large amount of chromium dioxide will be formed by reduction, so that the estimation of the content of adrenalin from the degree of the chrome reaction has thus been proved to be also theoretically correct. As the chrome reaction in the test-tube has shown, it is most distinct in a neutral medium; an acid or alkali medium disturbs the reaction. This explains why the chrome reaction in dead bodies is not proportional to the content of adrenalin, on account of postmortem changes which also affect the chemical reaction of tissues.

The above considerations explain why the chrome reaction in sections is most distinct in simple solutions of potassium dichromate. It can also be obtained with Mueller's fluid, Orth's fluid, dichromate-formalin solution, etc., because the other admixtures to these solutions do not disturb the reaction of the dichromate. In Zenker's

fluid, however, the chrome reaction becomes indistinct, not only because its constituent sublimate disturbs the reaction, but also because the large addition of acetic acid prevents the elimination of chromium dioxide.

As Henle has noted, the reaction can also be produced by chromic acid (Merck), but according to our observation, in the test-tube as well as in the sections, chromic acid must in this case be used only in a greatly diluted solution; 0.3 to 0.5 per cent is best. The reason is that the extracted chromium dioxide dissolves again in the abundant chromic acid. But microscopically the reaction caused by chromic acid is somewhat different on the slide. With chromic acid the atrabiliary part of the adrenals is of a diffuse brownish color, while with dichromate brownish granules appear in the protoplasm of the chromaffin cells. This may possibly be explained by the fact that the eliminated chromium dioxide is dissolved by the abundance of chromic acid, causing the diffuse coloration.

We have tried the reaction also with a solution of ammonium dichromate. The result was the same as with potassium dichromate.

The chrome reaction may also be effected by chromates as well as by dichromate and chromic acid. With chromates (we used potassium chromate), however, a large amount of adrenalin seems to be necessary for the reduction, for with the 1 per cent commercial adrenalin solution we could not produce the reaction in the test-tube, or in human materials, but it appeared distinctly in the fresh adrenals of healthy animals (rat, dog, pig). This reaction is similar to that of chromic acid,—microscopically it shows diffuse coloration.

We found that the chrome reaction appears best in the sections with 5 per cent solution of potassium dichromate, without any admixtures. But with this there is not sufficient hardening of the tissue, so we put the tissue first into 5 per cent potassium chromate solution for 6 to 12 hours, then added a tenth part of formalin, which gave the necessary hardening.

We also tried to combine the chrome reaction with subsequent coloring which some investigators have recommended for making the reaction more distinct. Such methods are Dewitzky's (25) (cresyl purple coloring) and Wiesel's (water blue safranin or toluidine blue safranin double coloring), but the simple toluidine blue coloring



recommended by Borberg (18) gave better results (discoloring in alcohol). We found that the grade of reaction can be estimated best without subsequent staining.

Since we have thus established that Henle's reaction is a simple reduction phenomenon of adrenalin upon chromic acid, especially dichromate, it follows that this phenomenon should not only occur with chromium compounds but also with other inorganic compounds. We have looked for such compounds, which should be easily reducible and insoluble in water. We first tried whether the reaction occurs in the test-tube, and if we obtained a precipitate we employed the medium also on tissues. But every reaction which succeeded in the test-tube was not always applicable to tissues. For instance, in a solution of potassium permanganate, manganese is at once precipitated by the addition of adrenalin, but because in tissues other organic substances besides adrenalin will reduce potassium permanganate, the adrenalin reaction is not recognizable by itself.

Fehling's solution, too, is reduced in the test-tube by adrenalin, but gave negative results in tissues. Fehling's solution contains a comparatively large amount of caustic soda, in consequence of which the adrenalin passes from the tissue into the solution and no copper oxide precipitates in the tissue.

We could easily reduce osmic acid by adrenalin in the test-tube. By using it on the adrenals the medulla becomes black but after this the lipoids of the cortex are also more markedly black. Afterwards we found that Mulon (15) in 1905 had observed the same phenomenon and also explained it as a reaction by adrenalin. He investigated the lipid of the adrenals and came upon this fact by accident.

We made experiments with nitrate of silver. A solution of it is easily reduced by adrenalin in the test-tube and silver is precipitated; in the tissue of the adrenal medulla also precipitation takes place, but some precipitation occurs in all parts of the tissue. not only in the medulla; moreover, the simple solution does not penetrate deep into the tissue. We have, therefore, made various experiments and shall describe our method.

After completing this investigation we found that a former investigator had already observed this silver reaction of the adrenals and likewise explained it as a reduction by adrenalin. Laignel-Lavastine (26, 27), in the course of extensive

histological investigations of the sympathetic nerves, also examined the nerve filaments of the adrenal medulla. He applied Kajal's silver impregnation on the adrenals of a rabbit and accidentally found a silver precipitate in the medullary cells. He believed that adrenalin was the cause of this phenomenon.

We also tried Kajal's method, but as we had found already with the simple nitrate of silver solution, silver is precipitated throughout the whole tissue and the method is therefore not applicable for the microscopic reaction of adrenalin.

In our own method we employed ammoniacal silver solution as follows: (1) Cut the adrenals (chromaffin tissue) with a sharp knife into sections as thin as possible; (2) place the sections without previous fixing in 1 per cent ammonium solution for 2 hours; (3) transfer to 5 per cent ammoniacal silver solution for 3 to 5 hours; (4) place again in 1 per cent ammonium solution, repeating this several times, for 30 minutes; (5) transfer to 3 per cent sodium thiosulfate solution for 1 hour (these operations, Nos. 3 to 5, must be made in the dark room, protected from daylight); (6) wash in running water for 1 hour; (7) fix in 10 per cent formalin solution; (8) prepare sections by any method.

If the reaction is positive the medullary cells are filled with black and silver granules. Silver granules were also found in blood vessels, which also occurs with the chrome reaction and indicates the presence of adrenalin in the blood vessels.

Da Costa (28) reported his application of Wilshowsky's silver method in the adrenals. According to an abstract, he obtained results similar to ours, but as we have not seen the original paper we do not know his method of procedure. Wilshowsky's method refers only to tissues fixed in formalin. A reduction by adrenalin cannot take place. We believe, therefore, that our silver method is different from Da Costa's.

#### SUMMARY.

We have established the fact that the chrome reaction as well as the silver and osmium reactions are merely reductions by adrenalin. In our opinion the naming of the cells giving a positive reaction should not be based upon the reaction (*i.e.*, chromaffin cells), but on the presence of adrenalin itself. Biedl's terms, adrenal cell, adrenal organ,

adrenal body, adrenal system, and also Bonnamour's term, adrenalin-producing cells, are appropriate in this respect. We propose the names adrenalin cell, adrenalin tissue, adrenalin system, thereby indicating the presence of adrenalin.

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## EXPLANATION OF PLATE 67.

Silver impregnation according to Ogata's method.

FIG. 1. Adrenal of pig.

FIG. 2. Human adrenal (beri-beri).

The chromaffin cells in the medulla of the adrenals are strongly impregnated.





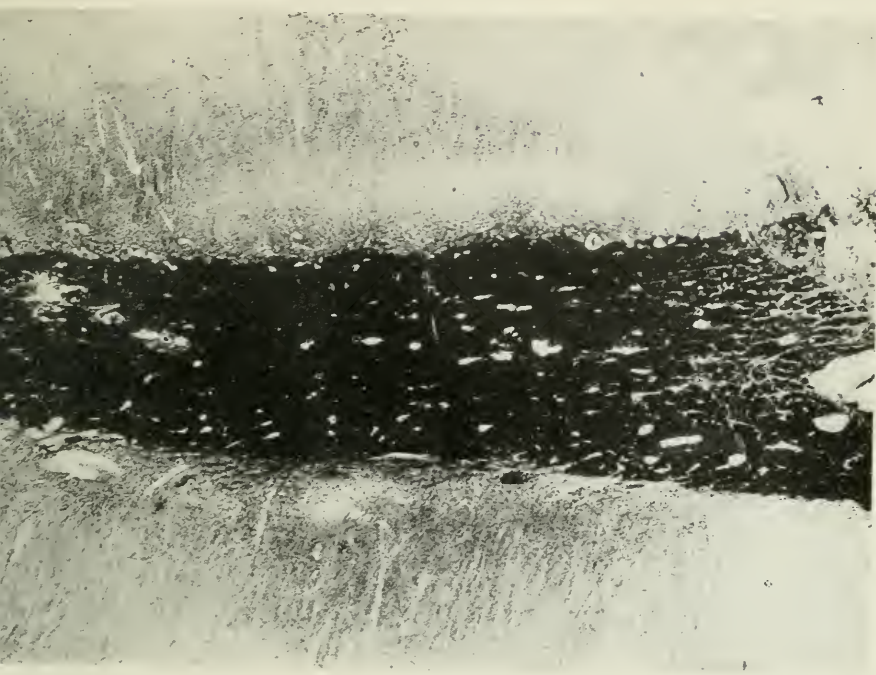


FIG. 1.



FIG. 2.

(Ogata and Ogata: Henle's Reaction of Chromaffin Cells.)



## STUDIES ON BACILLUS WELCHII WITH SPECIAL REFERENCE TO GAS GANGRENE.\*

By J. P. SIMONDS, M.D.

*(From the Department of Pathology, of Northwestern University Medical School,  
Chicago, and the Research Laboratory, Ambulance de l'Océan, La Panne,  
Belgium.)*

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Gas gangrene is preeminently an infection associated with war wounds. This is perhaps not due to any great difference in the degree of contamination of wounds received on the battle-field as compared with the injuries resulting from accidents in civil life. Spores of the group of bacteria capable of causing gas gangrene are probably no more abundant in the mud of the trenches than in ordinary street dirt, for they are known to have a wide distribution. The marked difference in the frequency of the disease in times of war and of peace is probably to be attributed to the dissimilarity in the character of the wounds encountered. In ordinary accidents, injuries associated with laceration and befouling of the tissues are usually too wide open to afford favorable conditions of growth to anaerobic bacteria which alone are able to cause typical gas gangrene. Shrapnel balls and, especially, fragments of highly explosive shells, on the other hand, produce penetrating wounds characterized by a comparatively narrow canal with ragged walls composed of lacerated tissues. Bits of clothing and other débris are frequently carried into the wound. The narrow channel becomes more or less completely filled with blood clot, and, thus sealed in, anaerobic bacteria are able to grow.

It must be remembered in this connection that these organisms enter the wounds in the form of spores. It is not improbable that spores of anaerobic bacteria require more favorable conditions for germination than vigorously growing vegetative forms demand for their continued multiplication. It is just these favorable conditions of an-

\* Aided by a grant from The Rockefeller Institute for Medical Research.

aerobiosis and suitable culture medium that are furnished in many war wounds.

In spite of the study of many sporadic cases of gas gangrene in civil practice, much remains to be learned concerning the pathogenesis of this infection. It was with the hope of discovering something as to the conditions essential to the occurrence of this disease in contaminated wounds that these studies were undertaken. As a preliminary investigation, the uniforms of a number of Belgian soldiers were examined to determine what percentage of them carried spores of this group of bacteria. Material—foreign bodies, lacerated tissues, etc.—removed from fresh wounds was then studied to find what proportion of these contained bacteria of this group. At the same time an effort was made to ascertain in what part of the wound germination of the spores was most prompt and complete. These patients were observed and the number that later developed gas gangrene was noted. It was also found convenient to study *in vitro* certain therapeutic measures employed in the treatment of wounds in general and of gas bacillus infections in particular.

*The Examination of the Uniforms of Belgian Soldiers for Spores of Anaerobic Bacteria Capable of Causing Gas Gangrene.*

The importance of the contamination of war wounds with bits of clothing in the causation of gaseous gangrene has been well recognized. Except for a few unpublished preliminary experiments by Dr. Alexis Carrel, I know of no systematic study of the uniforms of soldiers with reference to the presence of spores of bacteria capable of causing this disease. The examinations here reported were made in the Research Laboratory of the Ambulance de l'Océan at La Panne, Belgium. Unfortunately, on account of the difficulty of obtaining laboratory supplies in the War Zone, especially in a position so near the trenches as La Panne, it was impossible to test the virulence and pathogenicity of the organisms found or to do more than make the simple tests for group identification. The results of the work are presented, however, with the belief that they have some practical value.

These investigations were undertaken with three definite objects in view: (1) to determine the relative frequency with which the uniforms

carry spores of bacteria of the group known to cause gaseous gangrene; (2) to determine the efficiency of the sterilization of uniforms as practiced in the Belgian Army; and (3) to determine the time in which the uniforms become reinfected after the sterilization.

The materials studied consisted of the following: (1) The uniforms of twelve soldiers who had been in La Panne for 8 days to 3 weeks and away from the trenches for a slightly longer period. During this time their uniforms had been previously sterilized twice in four instances and once in each of the other eight. (2) The uniforms of thirty soldiers directly from the trenches. These were examined before and immediately after sterilization, and on each of the 3 succeeding days. During this time the men remained in La Panne. (3) Twenty new uniforms from the factory at Gravelines. (4) Samples of the cloth from which the uniforms were made.

The technique employed in the examination of the uniforms was simple. An area of approximately 5 sq. cm. on the outer surface of the blouse was selected, on the left side between the upper and lower pockets, and on the front of the trousers just above the knee. This area was washed several times with about 10 cc. of sterile water contained in a sterile Petri dish, over the edge of which the area of uniform was held. The water was drawn up several times in a sterile glass tube about 20 cm. long, and was allowed to flow repeatedly over the area under examination, the surface being rubbed at the same time with the end of the tube. 2 cc. of these washings were inoculated into tubes of whole milk recently sterilized in the autoclave. The tubes were then heated at 80°C. for 15 minutes to kill all vegetative forms of bacteria. The recent sterilization rendered the milk anaerobic, and the further heating at 80°C. expelled the air dissolved in the water used for inoculation. A sufficient degree of anaerobiosis was thus easily obtained for the growth of organisms of the group of *Bacillus welchii*.

The examinations before and immediately after sterilization of the uniforms were made at the Military Baths in La Panne,<sup>1</sup> under exactly similar conditions. This furnished a satisfactory control on

<sup>1</sup> For this privilege and for many courtesies we are indebted to Capt. Rolland, Officer in Charge of the Military Baths of the Belgian Army.



the method used. The results presented in the tables below indicate that there was no contamination due to faulty technique.

In examining the samples of cloth two methods were used: (1) The surface of the fabric was rubbed with a sterile swab moistened with sterile water. The swab was then placed in a tube of sterile anaerobic milk and heated to 80°C. for 15 minutes. (2) Small squares of the same size were cut from the cloth and passed through the free flame until the surface was thoroughly singed, or, in many cases, even charred. A series of control tests made by gently rubbing the burned surfaces of these squares with a moist sterile swab and inoculating broth and milk showed that the destruction of bacteria on the surface had been complete. The few cases in which these control tests showed that the surface sterilization was not perfect are not included in the tables given below. On scraping away the burned outer portion or on cutting across the square of cloth, the interior part of the goods was found almost unchanged. The singed or charred squares were placed in tubes of anaerobic milk and incubated either with or without previously heating to 80°C. for 15 minutes. Samples of cloth known to contain spores of *Bacillus welchii* were wrapped in gauze and placed in the pockets of uniforms ready to be sterilized at the Military Baths. After sterilization squares of these samples inoculated into anaerobic milk yielded no growth.

In all instances stained films from the milk cultures were examined. Inasmuch as the medium employed was especially favorable to the growth of the gas bacillus, very few other microorganisms were encountered in these studies. Those found were spore-forming bacteria which either (1) grew in cultures inoculated with washings which did not contain *Bacillus welchii*, or (2) were able to multiply in an acid medium and therefore grew in association with the latter organism.

The results of these examinations are shown in Tables I to IV.

It is seen from Table I that in this group 100 per cent of the trousers carried spores of anaerobic bacteria, and that more than 80 per cent contained spores of *Bacillus welchii*. The blouses were a little less frequently infected than the trousers. In the case of three of these garments no anaerobic bacteria grew in the cultures. Sterilization was thorough.

TABLE I.

*Examination of the Uniforms of Twelve Soldiers Who Had Been in La Panne on Coast Guard Duty for 8 to 20 Days.*

	Blouses.	Trousers.
Total number showing spores of anaerobic bacteria before sterilization.....	9	12
Total number showing spores of <i>B. welchii</i> before sterilization.....	9	10
Total number showing spores of bacteria of any kind after sterilization.....	0	0

TABLE II.

*Examination of Uniforms of Thirty Soldiers from the Trenches.*

Time of examination.	Garment.	Number with spores of anaerobic bacteria.	
		All kinds.	Group <i>B. welchii</i> .
Before sterilization.....	Blouses.	30	27
	Trousers.	30	27
Immediately after sterilization.....	Blouses.	0	0
	Trousers.	2	2
24 hrs.        "        " .....	Blouses.	7	7
	Trousers.	15	13
48 "        "        " .....	Blouses.	10	9
	Trousers.	20	20
3 days        "        " .....	Blouses.	14	11
	Trousers.	18	16

In Table II the first column of figures shows the number of garments containing spores of anaerobic bacteria of all kinds and therefore includes the figures in the second column. In this group of thirty uniforms, 100 per cent carried spores of anaerobic bacteria and 90 per cent had the spores of *Bacillus welchii*. The sterilization appeared not to be so effective in the case of these heavily contaminated garments as in the case of the previous group.

After the sterilization of their uniforms the soldiers remained in La Panne. The time of reappearance of the spores of *Bacillus welchii* upon these uniforms is also shown in Table II. It is evident that the spores of these bacteria were much less numerous on the uniforms 3

days after sterilization than when the men arrived from the trenches. It is on account of the comparatively slight recontamination that a garment which yielded a positive result on one day sometimes gave a negative one on the following day. With so few spores present it was possible to miss them. This is the explanation of the fact that, as shown in Table II, a larger number of trousers showed spores of *Bacillus welchii* 48 hours after sterilization than 72 hours after.

In many instances during the 3 days succeeding sterilization, 48 to 72 hours were required for the development of the typical reaction in milk inoculated with washings from the uniforms. Before sterilization this reaction developed in from 18 to 24 hours. This is indirect evidence of the comparative paucity of spores of the gas bacillus during the 3 days on which the examinations were made.

TABLE III.

*Examination of Twenty New (Unused) Uniforms.*

Number of cases in which spores of anaerobic bacteria were present..	{	Blouses.	9
		Trousers.	9
Number of cases in which spores of <i>B. welchii</i> were present.....	{	Blouses.	7
		Trousers.	9

It is seen from Table III that a notable percentage of the new (unused) uniforms carry spores of *Bacillus welchii*. Our results show less than 50 per cent so contaminated. It is probable, however, that these figures are too low. In the first place, they are much lower than the percentage of positive results obtained from an examination of samples of the cloth from which the uniforms were made (Table IV). Furthermore, the cloth of the new uniforms was so oily that it was impossible to bring the water into close contact with the goods. Any spores which may have been present, therefore, were dislodged with difficulty.

The samples of woollen cloth used in the experiments summarized in Table IV were obtained from the factory at Gravelines, and were identical with the cloth of the uniforms of the Belgian Army. The samples of cotton goods, few in number, came from the same source and represented the material used in making pockets and linings of uniforms. From this table it is evident that 100 per cent of the sam-

TABLE IV.

*Examinations of Samples of Cloth from Which the Uniforms Were Made.*  
*Total Number of Samples Examined: Wool, 48; Cotton, 10.*

Sample of cloth.	<i>B. welchii</i> .	Not sterilized.	Flamed.	Sterilized at baths.
Wool.....	Present.	23	14	0
	Absent.	0	3	8
Cotton.....	Present.	5	0	0
	Absent.	0	1	4

ples carried spores of *Bacillus welchii*. In the woollen goods the spores were in the meshes of the cloth. In the three instances in which negative results followed flaming of the sample, the process was prolonged until almost the whole mass was charred.

Spores of this organism are common in the gastro-intestinal tract of sheep and are constantly present in the soil. They were, therefore, undoubtedly on the wool when it was clipped from the animals. Whether these spores were not completely removed from the wool in the process of manufacture, or whether others were carried into the thread or woven cloth by the solutions used in dyeing or waterproofing, cannot now be stated.

It is evident from the above facts (1) that the new uniforms as received by the soldiers of the Belgian Army and the cloth from which these garments are made contain spores of *Bacillus welchii* in the meshes of the goods; (2) that 100 per cent of the uniforms of men from the trenches carry spores of anaerobic bacteria, and of these, 90 per cent carry the spores of the *Bacillus welchii* group; (3) that after sterilization of the uniforms, about 50 per cent of the trousers became contaminated with spores of the *Bacillus welchii* group during the first 24 hours, and about 66.66 per cent in the first 3 days when the men remain in barracks.

*The Presence of Bacillus welchii in Recent War Wounds in Relation to the Subsequent Development of Gas Gangrene.*

In the bacteriologic study of the uniforms of soldiers directly from the trenches, it was found that about 90 per cent carried spores of organisms morphologically like *Bacillus welchii* capable of causing

stormy fermentation of milk. The frequency with which bits of clothing are carried into wounds by fragments of shell and shrapnel balls has often been commented upon. It seemed desirable, therefore, to determine in what percentage of cases bacteria of this group could be isolated from fresh wounds, and to follow these cases in order to discover what proportion of them later developed gaseous gangrene.

Through the courtesy and cooperation of Dr. Depage, Surgical Director of the Ambulance de l'Océan at La Panne, it was possible to obtain material from twenty-one such cases. One of the patients, débris from whose wounds yielded *Bacillus welchii*, died of shock about 2 hours after reaching the Ambulance. The remaining twenty cases will therefore be made the basis of this report.

The material received for examination was removed by the surgeon under aseptic precautions and placed in sterile Petri dishes soon after the patient entered the hospital and, usually, within 2 to 8 hours after the reception of the wound. It consisted of bits of clothing and other débris, fragments of shell, lacerated tissues, and blood clots. Tubes of sterile anaerobic milk were inoculated, in duplicate in most instances, with pieces of the material. Duplicate cultures were made from muscle and from connective tissue (and fat) when these were removed from the wound. Since these bacteria were carried into the wounds in the form of spores, it was important to determine whether these spores had vegetated, and in what part of the wound and in what tissues this most readily occurred. One set of tubes thus inoculated was, therefore, heated to 80°C. for 15 minutes and then incubated. The other set of tubes was placed in the incubator without heating. In many cases the bacilli were isolated in pure culture and accurately identified.

Of the 20 cases, 5 proved entirely negative. Of these, only one contained pieces of clothing. In this case, unfortunately, only a heated culture was made. Of these 5 negative cases, none developed gaseous gangrene.

In the fifteen remaining cases, bacteria resembling *Bacillus welchii* were present. In ten of these, pieces of clothing were found in the wound; in the other five only fragments of shell and damaged tissues were removed.

Of the fifteen cases, three developed gaseous gangrene within the



first 48 hours after entering the hospital. The first of these was a young soldier with multiple shell wounds of the legs, on the service of Dr. Anten and Dr. Lagasse. He developed a mild localized gaseous gangrene around a wound about the middle of the right thigh. Under treatment, recovery was prompt. The other two cases were on the service of Dr. Van de Velde. In one of these a bullet entered the right thigh, shattered the femur, passed through the scrotum, and lodged in the muscles of the left thigh. The third case was a compound fracture of the right femur by a fragment of a hand grenade. Both of these patients developed severe gaseous gangrene of the right thigh, which was, however, controlled without resorting to amputation.

Stained films of the discharge from the wounds, in all twenty cases, were examined every 2 days. In the three cases which developed gaseous gangrene, microorganisms morphologically resembling *Bacillus welchii* were present in abundance at the beginning, but disappeared promptly after the subsidence of the gangrene. In two other of the fifteen positive cases a few bacilli similar to *Bacillus welchii* were seen in the first films examined, but were not present on subsequent examinations. In the remainder of the fifteen cases none of the films showed any bacilli that could be called *Bacillus welchii*.

In regard to the part of the wound in which vegetation of the spores is most prompt and complete, the two cases mentioned above on the service of Dr. Van de Velde yielded notable results. Pieces of damaged muscle and of connective tissue and fat were received from both cases in such a condition that separate cultures could easily be made. In both instances the heated and unheated milk tests were positive in the case of connective tissue and fat. In the case of the damaged muscle, however, the heated milk tubes remained sterile while the unheated duplicate tubes gave almost pure cultures of *Bacillus welchii*. Both of these patients developed gaseous gangrene. This same difference between injured muscle and connective tissue was also evident in other cases. From this, one would seem to be justified in concluding that the spores which are carried into the damaged muscle vegetate quickly.

This observation is important theoretically because it indicates the probable mode of origin of gaseous gangrene, and practically because

it suggests the prophylactic measures to be employed for its prevention. The spores which only reach the more superficial connective tissue are more exposed to the air and therefore find it difficult, if not impossible, to vegetate. In the more deeply lying muscle, on the other hand, a more perfect condition of anaerobiosis is attained. Furthermore, the exudate in the injured connective tissue is composed of lymph, blood, and blood serum; that is, a protein medium containing little or no sugar. In the lacerated muscle, on the contrary, one finds, in addition to the above exudate, the glycogen of the torn muscle fibers. Here, then, are the conditions favorable for the growth of *Bacillus welchii*; namely, anaerobiosis and a medium fairly rich in fermentable carbohydrate. It is in the injured muscle, therefore, that the spores first germinate and in which the gas, which is a most important factor in the spread of the bacilli and the advance of the infection in the tissues, is produced in abundance.

*Bacillus welchii* is not motile and cannot of itself change its position in the exudate of the infected area. It is a large heavy bacillus and is not readily transported through the narrow tissue spaces by the sluggish lymph. This organism produces substances which appear to be negatively chemotactic; and it is exceptional to find a bacillus in any of the few leukocytes present in the exudate in the wound. In the dissemination of the bacilli in the tissues adjacent to the wound it is the gas produced by them, the pressure of which may be very high,<sup>2</sup> that appears to be the most important factor. By forcing their way into the loose subcutaneous, inter- and intramuscular connective tissue, the bubbles of gas carry the bacilli with them, by the combined action of capillary attraction and surface tension, more and more deeply into the surrounding healthy tissues.

The onset and progress of gaseous gangrene may be visualized somewhat as follows. Bacteria capable of causing this disease are carried into the wound in the form of spores. Those which lodge in the more superficial connective tissue find conditions unfavorable to growth both as to the degree of anaerobiosis and as to the character of the culture medium. Those which are carried into the more deeply placed lacerated muscle find conditions perfectly adapted to their

<sup>2</sup> Taylor, K., *Lancet*, 1916, i, 123.

needs and therefore germinate and multiply rapidly. Gas is produced in abundance from the glycogen of the crushed and torn muscle fibers. The bacilli are forced along the loose connective tissue by the infiltrating gas among the more healthy muscle bundles, and the infection advances. The rapidity of its progress depends in part, at least, upon the amount and pressure of the gas and the looseness of the connective tissue.

It is difficult, and usually impossible, to obtain growth of *Bacillus welchii* in pure protein media such as sugar-free broth or sometimes even in plain broth, unless large quantities of an actively fermenting culture are used for inoculation. This is especially true if other bacteria are present. In such a favorable medium as milk or dextrose agar, on the other hand, there is evidence that very small numbers of vegetative forms or spores of this microorganism, perhaps even single spores,<sup>3</sup> will multiply rapidly, and easily outgrow other bacteria present. An almost equally favorable medium is found in lacerated muscle tissue. One may therefore conclude that when the spores have once been carried into the wound, the most important element in their germination and the onset of the infection is the damaged muscle with its anaerobiosis and fermentable carbohydrate. When the spores have vegetated and vigorous growth begins, the important elements in the spread of the infection are the pressure of the gas and the loose connective tissue. The further rapid growth of *Bacillus welchii* in the wound may be due either (1) to the heavy inoculation of the more purely protein exudate of the connective tissue from the growth in the muscle; or (2) to the further liberation of glycogen from other muscle fibers killed by the action of the acid products of fermentation or by pressure of the gas. That the latter is more probable is supported by the histologic studies of Taylor.<sup>4</sup>

In the various attempts to explain the pathogenesis of gaseous gangrene much emphasis has been laid upon the fact that nearly all cases of this infection occur in the extremities,<sup>5</sup> especially the lower. These

<sup>3</sup> Simonds, J. P., Monograph of The Rockefeller Institute for Medical Research, No. 5, 1915, 34, 85.

<sup>4</sup> Taylor, J. *Path. and Bacteriol.*, 1915-16, xx, 384.

<sup>5</sup> For a full bibliography, with brief abstracts, of papers which have appeared in French and English journals, see Moiroud, P., and Vignes, H., *La gangrene gazeuse, et les plaies gangreneuses*, Paris, 1916.

are the locations in which large masses of muscle are found. Of the cases in this series which showed *Bacillus welchii* in the fresh wounds but failed to develop gaseous gangrene, none were wounds involving injury to large masses of muscle. They were either superficial injuries, or wounds of the head, face, trunk, joints, or feet where muscle tissue is not very abundant.

Summarizing these results, it was shown that in this series of twenty cases, 75 per cent of the fresh wounds contained spores or vegetative forms of bacilli morphologically resembling *Bacillus welchii* and capable of producing stormy fermentation of milk. In most cases the organisms were isolated in pure culture and identified. Only 20 per cent of this number, *i.e.*, 15 per cent of the whole series, developed gaseous gangrene. It is only in the injured muscle that these spores find conditions favorable to immediate germination and rapid multiplication; namely, anaerobiosis and a culture medium rich in fermentable carbohydrate. In the pathogenesis of this infection in contaminated wounds, therefore, the lacerated muscle is the most important factor, and it is to this that attention should be directed in the prevention of the disease.

*The Influence of Different Concentrations of Saccharose upon the Growth of Bacillus welchii.*

It is well known from the work of Theobald Smith<sup>6</sup> and of Kendall<sup>7</sup> and his coworkers that the presence of fermentable carbohydrate in culture media favors the growth and profoundly modifies the metabolism of many bacteria. Concentrated solutions of sugars, on the contrary, either kill most bacteria or inhibit their growth. It was probably these two principles which led to the use of sugar in the treatment of war wounds. In the case of wounds infected with ordinary pathogenic bacteria this method of treatment may have a reasonable theoretical basis. In the case of wounds contaminated with spores of *Bacillus welchii*, however, one would have good reason to fear that the application of sugar might bring disastrous results.

<sup>6</sup> Smith, T., *Tr. Assn. Am. Phys.*, 1896, xi, 37.

<sup>7</sup> Kendall, A. I., *J. Bio' Chem.*, 1912-15. Kendall, A. I., and Walker, A. W., *Tr. Chicago Path. Soc.*, 1915, ix, 320.



This microorganism can be made to grow only with difficulty in sugar-free media. In media containing almost any carbohydrate, however, growth is surprisingly rapid,—at times almost explosive in character. It is probable that none of this group of bacteria produce a soluble toxin; certainly not in the presence of fermentable sugar. This group is distinctly not putrefactive. It is purely fermentative. It is from sugar that they produce irritating and injurious substances in the form of butyric and other organic acids.<sup>8</sup> It has been noted that in a series of fresh wounds it was found that 75 per cent contained either the spores or the vegetative forms of *Bacillus welchii*. For this reason it seemed desirable to study experimentally the effect of different concentrations of sugar upon the multiplication and gas production of this bacillus.

Ten strains of *Bacillus welchii* isolated either from distinct cases of gaseous gangrene or from débris found in fresh wounds were selected for study. Bouillon containing 5, 10, 20, 40, 50, and 60 per cent respectively of saccharose was sterilized in the autoclave in fermentation tubes and in ordinary test-tubes under oil, cooled, and inoculated.

Three methods of inoculation were employed: (1) tubes of all concentrations were inoculated from a 24 hour milk culture; (2) subcultures in the 5 per cent saccharose broth were made from a milk culture, incubated for 24 hours, and inoculations were made from these into the higher concentrations; (3) transplants were made from a 24 hour culture in each dilution into the next higher; that is, from 5 per cent into 10 per cent, from 10 per cent into 20 per cent, and so on through the series. The results were the same with all three methods. During the period covered by these experiments there was no visible tendency for any of the strains gradually to become accustomed to growth in the presence of high concentrations of sugar. In a few instances there was a small amount of gas in tubes of 50 per cent saccharose inoculated from a 24 hour milk culture, while no gas was found in a duplicate 50 per cent tube inoculated from a 24 hour culture of the same strain in 40 per cent sugar broth.

These experiments were repeated a number of times with different lots of media but with the same concentrations of sugar. Characteris-

<sup>8</sup> Herter, C. A., *The Common Bacteriological Infections of the Digestive Tract and the Intoxications Arising from Them*, New York, 1907.



tic results are shown in Table V in which the amount of gas produced in the closed arm of a fermentation tube has been measured in the usual manner. Since the inoculations were always made from one fluid culture into another of higher specific gravity, the organisms carried over in inoculating rose immediately to the top of the closed arm of the fermentation tube where the condition of anaerobiosis was ideal.

TABLE V.

Culture.	Saccharose.					
	5 per cent.	10 per cent.	20 per cent.	40 per cent.	50 per cent.	60 per cent.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
617 c	70	60	80	40	0	0
669 b	70	90	90	60	5	0
285	80	65	90	50	60	0
365 a*	85	70	90	85	Trace.	0
365 b*	85	70	90	60	0	0
793 a*	70	80	75	80	0	0
793 b*	—	80	—	40	60	0
797 a	70	100	80	75	10	0
"Central"	40	95	95	70	Trace.	0
386 cd	70	60	55	85	0	0

\* 365 a and 365 b were fished from separate colonies in the original cultures from the same case. The same is true of 793 a and 793 b.

From this table it is evident that *Bacillus welchii* can produce an abundance of gas in broth containing amounts of saccharose up to 400 gm. per liter. Some strains are able to ferment this sugar in 50 per cent concentration. Of the ten strains studied, not one was able to produce gas in 60 per cent saccharose broth. The bacteria in these cultures were not always dead, for in many instances anaerobic milk, inoculated from 60 per cent saccharose broth tubes that had been in the incubator for 3 days, showed stormy fermentation. It was observed that in the dilutions up to 20 per cent, fermentation was very rapid and was practically complete in 24 hours. Production of gas proceeded much more slowly in the higher concentrations. At the end of 24 hours there was only a small bubble of gas in the 40 per cent tubes and in the 50 per cent tubes which showed any gas production. The process required 48 to 72 hours for completion. In a few instances

there appeared to be some growth of the bacilli in 50 per cent saccharose broth, as evidenced by the increased turbidity in the upper part of the closed arm of the fermentation tube, without the production of gas.

Stained films were made after 24 to 48 hours and examined to determine the effect upon morphology of placing these bacilli in solutions of such high specific gravity as the 50 and 60 per cent saccharose solutions. Except for a possible slight increase in thickness and a tendency to stain palely, there were no detectable morphologic differences between bacilli grown in the higher concentrations of the sugar and those grown in the weaker concentrations.

Because the presence of fermentable sugar is essential to the vigorous growth of *Bacillus welchii*, and because saccharose has been used in the treatment of war wounds, at least 75 per cent of which are contaminated with spores of this organism, it seemed desirable to study the effects of different concentrations of sugar upon its growth. All the strains studied grew and produced an abundance of gas in broth containing 5, 10, 20, and 40 per cent respectively of saccharose. In the 40 per cent solution formation of gas was somewhat slower than in the lower concentrations. Only two strains produced gas in abundance, somewhat slowly, in 50 per cent saccharose broth. None of the strains grew or produced gas in broth containing 60 per cent saccharose.

#### *The Effect of Pure Oxygen Gas on Cultures of Bacillus welchii.*

Injections of pure oxygen gas directly into the tissues is used as a routine treatment for gas gangrene in many of the military hospitals in France and Belgium.<sup>9</sup> In a previous study<sup>10</sup> of *Bacillus welchii* it was observed that the degree of anaerobiosis in milk cultures influenced materially the type of reaction of these organisms in this medium. Variations were noted from no growth in milk saturated with

<sup>9</sup> Vennin, Girode, and Haller, *Bull. et mém. Soc. chir., Paris*, March 9, 1915; for further references and abstracts see Moiroud and Vignes, *La gangrene gazeuse*, Paris, 1916.

<sup>10</sup> Simonds, Monograph of The Rockefeller Institute for Medical Research, No. 5, 1915, 36.

air, to coagulation with little or no gas formation in tubes containing small amounts of air, and to typical stormy fermentation in milk with the optimum degree of anaerobiosis. It seemed possible, therefore, that the efficacy of the injections of oxygen might not depend solely on their supposed bactericidal action. A study of the effect of pure oxygen gas upon cultures of *Bacillus welchii* was undertaken.

The following technique was employed. Tubes of anaerobic milk or dextrose broth under oil were inoculated with *Bacillus welchii*. Oxygen was passed into the medium by means of a capillary pipette which reached to the bottom of the tube. The gas rose through the liquid in a series of many minute bubbles. After oxygenation the tubes were placed in the incubator, and at intervals subcultures were made in fresh anaerobic milk or deep dextrose agar by transferring about 0.5 cc. of the culture into the new medium. In another series of experiments tubes of milk or dextrose broth were inoculated with *Bacillus welchii*, incubated for 3 or 4 hours, and then oxygenated for 20 minutes as described above. Typical results from numerous experiments are collected in Table VI.

It is evident from Table VI that strains of *Bacillus welchii* differ in their resistance to oxygen. Strains 617 a and 617 ds are much more readily killed by oxygen than Strain 365 a. Whether the resistance is in inverse proportion to the virulence of the organism cannot be stated. But Strain 365 a was isolated from a mild localized case of gas gangrene, and when its pathogenicity for guinea pigs was tested for the first time about 3 months after its isolation, it was found to be wholly non-virulent. Strain 617 a, on the other hand, was obtained from a severe case of gas gangrene, and after 3 months' growth on artificial media caused the death of a guinea pig in 24 hours.

When tubes of milk inoculated with *Bacillus welchii* were incubated for 3 hours before the oxygen gas was bubbled through them, the oxygenation did not appear to inhibit their growth in any way. Even Strain 617 a, unincubated cultures of which were sterile in less than 2 hours after passage of the gas for 20 minutes, was not affected by 20 minutes' oxygenation if the culture had been previously incubated for 3 hours.

In instances in which the organisms were not actually killed by the oxygen it will be noted that there was a marked reduction in the

TABLE VI.

Culture.	Time in incubator before oxygenation.	Time of passage of oxygen gas.	Original culture after incubation.			Subcultures made after			
			24 hrs.	48 hrs.	72 hrs.	2 hrs.	4 hrs.	6 hrs.	24 hrs.
	hrs.	min.							
365 a	0	5	++++			++++		++++	
365 a	0	10	+++			++++		+++	
365 a	0	20	+++			++++		+++	
365 a	3	20	++++						
386 cb	0	5	+++			++		+	++++
386 cb	0	10	-	-	+	++		+	+
386 cb	0	20	-	-	+	++		+	+
386 cb	3	20	++++						
617 ds*	0	5	+++			++		++	
617 ds	0	10	-	-	-	+		-	-
617 ds	0	20	-	-	-	+		-	-
617 ds	3	20	++++						
617 a*	0	10	-	-	-	-	-	-	
617 c*	0	10	+++			+++		+++	
617 c	0	15	-	-	-	+	+	-	-
553 a	0	5	-	+	+	++	+		+
553 a	0	10	-	-	-	++	-	-	-

++++ indicates typical stormy fermentation; +++, coagulation with less gas than typical stormy fermentation; ++, coagulation with small amount of gas; +, coagulation with no gas; -, no change in medium.

\* Cultures 617 a and 617 c are two strains isolated from the same case of gas gangrene. Strain 617 ds is Strain 617 a after it had been passed through a guinea pig in which it produced a fatal gas gangrene.

production of gas in the culture. This may have been due in part to the restraining effect of the oxygen on the multiplication of the bacilli so that there were too few organisms present to produce the usual amount of gas. But this same character of depressed gas formation was carried on into subsequent generations. Films from all these cultures showed evidence of considerable growth, although it was not possible to estimate accurately the relative amount in the different tubes. It seems, therefore, that exposure to oxygen may depress the biologic activity of gas bacilli without actually causing their death. This lowered activity can be carried on through a number of generations even when grown in as favorable a medium as milk. It is

not associated, however, with the acquirement of any new or strange characters such as ready spore formation or liquefaction of gelatin. These depressed cultures can be easily rejuvenated by growth for a generation or two in anaerobic dextrose-liver-broth medium.

It might be mentioned that in studying organisms of this group, no biologic reactions should be carried out except with recently rejuvenated cultures. Only in this way can uniform and characteristic results be obtained.

From the experiments described above one may suggest as practical possibilities (1) that injections of oxygen gas will be much more effective if made early in the course of gas gangrene than if delayed until the growth of the infecting organisms has acquired considerable energy and momentum; and (2) that the good effects of the injection of oxygen may not be due wholly to its bactericidal effects but partly to its power to depress the vital activity of *Bacillus welchii* whereby less gas is produced.

#### SUMMARY.

1. Spores of the *Bacillus welchii* group of bacteria were found on 100 per cent of the uniforms of Belgian soldiers who had come directly from the trenches, and in the meshes of all the samples examined of the new cloth from which the uniforms were made.

2. In fifteen out of twenty fresh war wounds members of this group of bacteria were found. Of the fifteen patients, only three later developed gas gangrene. Once the spores of *Bacillus welchii* have been carried into a wound the deep-lying lacerated muscle tissue appears to be the most important factor in the onset of gas gangrene.

3. *Bacillus welchii* is able to grow and produce gas in broth containing up to 40 per cent saccharose. Some strains were able to multiply and produce gas in 50 per cent saccharose broth; but none of those examined were able to grow when the concentration of the sugar reached 60 per cent.

4. The bubbling of pure oxygen through milk or dextrose broth cultures of *Bacillus welchii* has a definite depressor action on the production of gas. This does not appear to be due to a reduced number of organisms in the culture.



## EQUILIBRIA IN PRECIPITIN REACTIONS.

### THE COEXISTENCE OF A SINGLE FREE ANTIGEN AND ITS ANTIBODY IN THE SAME SERUM.\*

By STANHOPE BAYNE-JONES, M.D.

*(From the Department of Bacteriology of the College of Physicians and Surgeons,  
Columbia University, New York.)*

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The vagueness of the terms of serology connotes the difficulties with which the describers of this new knowledge have had to contend. Unable to use simple and definite chemical substances, and without the guidance of any general theory, they have had to employ mass adjectives to describe the reactions of a serum as a whole, and have gathered great numbers of observations without any plan for their coordination. Whenever, therefore, it is possible to repeat former experiments by substituting known factors in place of unknown mixtures, the work should be undertaken. Quantitative data will be obtained through these studies which will eventually allow serology to find a place in either chemistry or physics, or what seems almost self-evident, in physical chemistry. The colloidal state of matter has afforded so many analogies to the reactions of serology that the terms of colloid chemistry have already been applied with uncritical facility by immunologists to reactions which have not yet been studied with quantitative accuracy sufficient to indicate their true nature. The systematic attempt, however, to prove or disprove these colloidal analogies in serology will undoubtedly result in a unifying conception of the principles of the reactions of immunity.

The precipitation of protein by specific sera has many analogies with colloidal reactions and this phenomenon lends itself readily to investigation simplified by the use of at least a single pure substance.

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Zinsser<sup>1</sup> has emphasized the colloidal phases of the precipitin reaction, and recently Weil<sup>2</sup> has repeated the experiments, using crystallized egg albumin as the antigen. One of the phases of the precipitin reaction which has engaged particular attention is that in which the precipitinogen and the precipitin occur simultaneously but ununited in the same serum. This is easily demonstrable when whole serum or a mixture of proteins is used as the antigen. Weil, however, using crystalline egg albumin as the antigen was unable to demonstrate the coexistence of free antigen and antibody in the same serum. It is of considerable theoretical importance to confirm this finding. The results obtained by Weil are so little in accord with expectations based upon chemical or colloidal theories that it was considered advisable to repeat the experiments on precipitin reactions with crystalline egg albumin. The following studies, therefore, were carried out under the direction of Dr. Zinsser, with some modifications of recent work on this reaction.

#### HISTORICAL.

In 1902 Linossier and Lemoine,<sup>3</sup> Ascoli,<sup>4</sup> and Eisenberg<sup>5</sup> noticed that when foreign serum had been injected into rabbits in large doses the sera of these animals contained both antigen and antibody. They used whole sera as antigens. To demonstrate the simultaneous presence of free precipitable substance and its precipitant in the same serum, fractions of the serum were tested, first for antigen, by adding another antiserum, and second for precipitin by adding an homologous antigen. Gay and Rusk<sup>6</sup> have described the same phenomena, and by showing that the sera do not fix complement, although they contain antigen and antibody, they have furnished additional evidence of the ununited state of the substances.

Zinsser and Young<sup>7</sup> have pointed out that the association of free antigen and its antibody in serum, as shown by the failure of this serum to fix complement, is evidence that the reaction does not take place according to the law of mass

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<sup>1</sup> Zinsser, H., *Infection and Resistance*, London and New York, 1914, 266.

<sup>2</sup> Weil, R., *J. Immunol.*, 1916, i, 19.

<sup>3</sup> Linossier, G., and Lemoine, G.-H., *Compt. rend. Soc. biol.*, 1902, liv, 85.

<sup>4</sup> Ascoli, M., *Münch. med. Woch.*, 1902, xlix, 1409.

<sup>5</sup> Eisenberg, P., *Centr. Bakteriöl., 1te Abt., Orig.*, 1903, xxxi, 773.

<sup>6</sup> Gay, F. P., and Rusk, G. Y., *Univ. California Publications, Pathology*, 1912, ii, 59, 73.

<sup>7</sup> Zinsser, H., and Young, S. W., *J. Exp. Med.*, 1913, xvii, 396.

action. According to the formula of that law, this serum should not only contain free antigen and antibody, but also a certain quantity of the united complex. To explain the inhibition of the union of antigen and antibody in such sera Zinsser and Young<sup>7</sup> have drawn a strict parallel with the protective colloidal reactions. These experiments show that it is not improbable that the serum proteins act as protective colloids, preventing the precipitation of a protein by its antiserum, just as gum arabic will protect colloidal arsenic sulfide against the precipitating action of gelatin.

Von Dungern<sup>8</sup> confirmed the findings of Linossier and Lemoine but explained them according to a different hypothesis. He showed that whole serum consists of a number of protein complexes, all of which cause the formation of precipitin in the immunized animal. One portion of the protein mixture may have more antigenic properties than another, as a result of which partial precipitins of various strengths are produced for the whole serum. Von Dungern believed that these complex antigens and multiple antibodies reacted irregularly in such a way that while the supernatant fluid of a precipitin test would contain free antigens found in part in the original serum together with precipitins capable of flocculating the original serum, yet the antistances in the supernatant fluid were not strictly homologous and hence would not react with each other. His extensive experiments have had great influence upon subsequent opinions. He worked, however, entirely with multiple antigens and gave only inferences and analogies for the precipitin reactions with pure proteins.

At first glance it would seem as if the experiments in the precipitation of casein with lactoserum, reported by Müller,<sup>9</sup> were evidence that with purified antigens the phenomenon of Linossier and Lemoine did not occur. The supernatant fluids after his precipitin reactions never contained free antigen along with antibody. His experiments, however, are not relevant to the results of precipitin reaction with purified protein. Müller used whole milk as his casein, obviously a multiple antigen, and the degrees of dilutions used in the tests leave such large gaps that the zone in which antigen and antibody coexist ununited might have been overlooked.

Weil<sup>2</sup> confirms all the previous observations on the coexistence of free antigen and antibody in the supernatant fluids of precipitin tests when the whole serum, raw egg white, or any mixed protein is used. With purified egg albumin as antigen, the results were different. He states: "If this antigen is mixed in graded proportions with the serum of a rabbit immunized thereto, and the resulting precipitates are removed by centrifugation, the supernatant fluid never contains both antigen and antibody; either one is present alone." The protocols of Weil's precipitin tests allow him to draw sharp conclusions between the effect of 0.005 cc. and 0.004 cc. of the antigen, a delicacy of reaction which not all workers have

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<sup>8</sup> von Dungern, F., *Centr. Bakteriolog., 1te Abt., Orig.*, 1903, xxxiv, 355.

<sup>9</sup> Müller, P. T., *Centr. Bakteriolog., 1te Abt., Orig.*, 1903, xxxiv, 48.

been able to attain. The crystalline egg albumin, prepared for him by Coca, was certainly as pure as it is possible to obtain this substance. It is not stated whether anaphylactic reactions were carried out with this particular preparation to determine whether it was thereby free from antigen complexes which cause reactions in animals sensitized with egg globulin. Weil,<sup>2</sup> however, referring to his earlier work with Coca<sup>10</sup> states that he "found that if egg albumin and egg globulin were separated by chemical means, and different rabbits were immunized to each substance, the resulting immune sera reacted powerfully each with its own antigen, and very weakly with the other antigen." This anaphylactic interrelation of egg proteins will be discussed later. It is significant here to note the apparent discrepancy between the results of these precipitin tests and anaphylactic reactions, although one antigen was used which admittedly gives some anaphylactic reaction with other proteins.

#### EXPERIMENTAL.

Sera capable of precipitating egg albumin were prepared by immunizing rabbits with intravenous injections of this protein. By varying the proportions of immune serum and solution of egg albumin in the precipitin tests, zones of dilution were obtained in which tests for free antigen and free antibody could be made. In addition, by testing the sera of immunized rabbits at certain intervals after the injection of albumin, the proportions and duration of circulating antigen and antibody could be studied.

The important material for these experiments is the antigen. Attempts to use casein failed because of the confusing precipitates caused by the calcium salts required in the tests with this protein. In some experiments, pure edestin from hemp-seed was used.<sup>11</sup> The results with this protein were definite. Because, however, of the slight solubility of edestin, work with it is somewhat difficult. Crystalline egg albumin was found to be most suitable for these studies. This substance was prepared<sup>12</sup> according to the method of Hopkins and Pinkus.<sup>13</sup> The whites of freshly laid eggs were carefully precipitated with ammonium sulfate and the albumin from these was

<sup>10</sup> Weil, R., and Coca, A. F., *Z. Immunitätsforsch., Orig.*, 1913, xvii, 141.

<sup>11</sup> The preparation was made by Dr. Harris of the Connecticut Agricultural Experiment Station.

<sup>12</sup> The substance was prepared under the direction of Mr. Balls of the Department of Physiological Chemistry of Columbia University.

<sup>13</sup> Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898-99, xxiii, 130.



recrystallized three times. The solution of protein was dialyzed under toluene in the ice box to free it as much as possible from ammonium sulfate and acetic acid. The albumin was then dried *in vacuo* to a white glistening powder. The Zsigmondy "gold number"<sup>14</sup> for the preparation was 8, showing the usual protective value of pure egg albumin as a colloid. This preparation met the requirements for pure crystalline egg albumin and was probably as pure as it is possible to obtain a protein of this nature.

Anaphylactic tests were made in the hope of showing that this preparation of egg albumin was free from globulin or substances which had antigenic properties like globulin. Egg globulin was prepared from the first fraction of egg white precipitated by half saturation with ammonium sulfate. A solution of the precipitate was dissolved in distilled water and dialyzed against distilled water until the globulin began to flocculate. This material was centrifuged and the precipitate taken up in 10 per cent sodium chloride. This formed a turbid emulsion, free, however, from large particles. One series of guinea pigs weighing 250 gm. was sensitized by intravenous injections of varying amounts of crystalline egg albumin; another series by intravenous injections of egg globulin. After an interval of 26 days the animals were reinjected with protein (Table I).

The results of the experiments summarized in Table I show that preparations of egg albumin and egg globulin have a common antigenic factor, capable of producing mutual anaphylactic reactions. They confirm the statements of Wells<sup>15</sup> and Wells and Osborne,<sup>16</sup> who, after a long series of experiments concluded that: "In spite of the most careful separation of these two portions of egg white by means of ammonium sulfate precipitation, the resulting preparations each react almost as well against the other as against itself." Weil and Coca<sup>10</sup> report a somewhat similar experience with crystalline egg albumin when injected into animals sensitized to egg globulin. These reactions have been considered in detail in order to determine whether or not the anaphylactic test is a valid criterion of the purity of a pro-

<sup>14</sup> Schulz, F. N., and Zsigmondy, R., *Beitr. chem. Phys. u. Path.*, 1903, iii, 137.

<sup>15</sup> Wells, H. G., *J. Infect. Dis.*, 1911, ix, 147.

<sup>16</sup> Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1913, xii, 341.



TABLE I.

*Anaphylactic Reactions with Solutions of Ovalbumin and Ovoglobulin from the Same Eggs. Guinea Pigs, Weight 200 to 250 Gm., Injected Intravenously on July 13, 1916. Second Injection on August 7, after an Interval of 26 Days.\**

Guinea pig No.	Sensitizing dose.	Interval.	Second injection.	Result.
Animals sensitized to crystalline ovalbumin.				
		days		
1	0.002 gm. of albumin.	26	0.1 gm. of albumin.	Convulsions. Died.
2	0.002 " " "	26	0.2 " " "	" " in 2 hrs.
3	0.001 " " "	26	0.1 " " "	Typical anaphylaxis. Died in 3 min.
4	0.001 " " "	26	0.1 " " "	Convulsions. Died in 3 min.
5	0.0005 " " "	26	0.1 " " "	Violent convulsions. Died in 2 min.
6	0.002 gm. of albumin.	26	0.1 gm. of globulin.	Convulsions. Died in 3 min.
7	0.002 " " "	26	0.1 " " "	Died in 30 min.
8	0.001 " " "	26	0.002 " " "	Diarrhea. Survived.
9	0.001 " " "	26	0.002 " " "	Sneezing, coughing. Survived.
Animals sensitized to ovoglobulin.				
10	0.004 gm. of globulin.	26	0.1 gm. of globulin.	Convulsions. Died in 1 min.
11	0.002 " " "	26	0.002 " " "	" " " "
12	0.002 " " "	26	0.002 " " "	" Paralysis in hind legs. Died in 2 min.
13	0.001 " " "	26	0.002 " " "	Cough. Survived.
14	0.001 " " "	26	0.002 " " "	Convulsions. Died in 2 min.
15	0.0005 " " "	26	0.002 " " "	" " " "
16	0.002 gm. of globulin.	26	0.1 gm. of albumin.	Cough. Survived.
17	0.002 " " "	26	0.1 " " "	Sneezing, dyspnea. Survived.
18	0.001 " " "	26	0.1 " " "	Sprawling, twitching. Survived.
19	0.001 " " "	26	0.1 " " "	Cough. Survived.
20	0.0005 " " "	26	0.1 " " "	" "

\* Separate syringes and needles were used for each protein.

tein, and in particular whether the anaphylactic interreactions of egg globulin and egg albumin exclude the latter from use as a single pure antigen. Crystalline egg albumin is acknowledged to be a pure protein.<sup>15,17</sup> Egg globulin, however, probably consists of ovomucin and ovalbumin admixed in indefinite proportions.<sup>15</sup> The impurity of the globulin renders the anaphylactic reactions without significance. In the protocols of Table I, it is seen that the albumin-globulin reactions are much weaker than those caused by the injection of the specific protein into a sensitized animal. This undoubt-

## EXPERIMENT I.

*Edestin as Antigen.*

This experiment was done to show coexistence of edestin and its precipitin in the serum of an immunized rabbit.

Apr. 15-18, 1916. Serum from Rabbit 21, immunized to edestin, obtained 1 hour after an intravenous injection of 12 cc. of 0.027 per cent solution of edestin. This serum was kept for 3 days in the ice box before being tested for its content of antigen and antibody. Serum from Rabbit 22 also immunized to edestin, having a precipitin titer of 1:4,000. These sera were mixed in various proportions. The diluent was a mixture containing 0.9 per cent sodium chloride and 0.105 per cent sodium carbonate in order to keep the edestin in solution.

Tube.	Serum 21.				Serum 22.		Precipitate.		
	Dilution.	Amount.	Edestin.	Chloride-carbonate solution.	Dilution.	Amount.	Ring.	After 4 hr. at 37°C.	After 24 hrs. in ice box.
For precipitin.		cc.	cc.	cc.		cc.			
1	Undiluted.	0.2	0.2	0.5			±	+	+
2	1 : 5	0.2	0.2	0.5			o	++	++
For antigen.									
3	Undiluted.	0.2		0.5	Undiluted	0.2	+	++	++
4	1 : 100	0.2		0.5	"	0.2	++	+	+
5	1 : 1,000	0.2		0.5	"	0.2	±	±	±
Controls.									
6	Undiluted.			0.7			o	o	o
7				0.7	"	0.2	o	o	o
8			0.2	0.7			o	o	o
9			0.2	0.5	1:4,000	0.2	±	+	+

<sup>17</sup> Schryver, S. B., General Characters of the Proteins, New York, 1909, 20.

edly indicates that the globulin preparation contained ovalbumin. Belief in the individuality of the crystalline egg albumin as an antigen rests upon its chemical characteristics.

Accepting, therefore, purified edestin and ovalbumin as single antigens, the experiments were conducted upon their specific precipitin reactions.

#### EXPERIMENT II.

##### *Edestin as Antigen.*

Apr. 20, 1916. Various quantities of edestin were added to its antiserum to find the zone of dilution *in vitro* in which antigen and antibody occur in supernatant fluids after the primary precipitin reaction.

Serum from Rabbit 22, immunized to edestin, having a titer of 1:2,000.

0.02 per cent solution of edestin mixed in various proportions with undiluted Serum 22.

Tube.	Serum 22, undiluted.	0.02 per cent solution of edestin.	Chloride- carbonate mixture.	Precipitate after 24 hrs. in ice box.	Supernatant fluids.	
					For antigen.	For precipitin.
					0.2 cc. of supernatant fluid plus 0.3 cc. of Serum 22.	0.2 cc. of supernatant fluid plus 0.3 cc. of edestin solution.
	cc.	cc.	cc.			
1	0.2	2		++	++++	o
2	0.2	1		++	++	±
3	0.2	0.5	0.3	+++	++	±
4	0.2	0.2	0.6	+++	+	+
5	0.2	0.1	0.7	++	Tr.	+
6	0.2	0.01	0.8	+	?	+
7	0.2	0.001	0.8	+	o	++
8	0.2	0.0001	0.8	±	o	++
9	0.2	0.00001	0.8	o	o	++
Controls.						
10	0.2		0.3	o	o	++
11		0.2	0.3	o	++	o

This experiment demonstrates the phase in the precipitin reaction in which a single antigen occurs simultaneously but ununited with its homologous antibody. In the table, the shaded columns overlap each other through the zone in which edestin and antiedestin were found free in the same fluid.

## EXPERIMENT III.

*Crystalline Egg Albumin as Antigen.*

June 15-16, 1916. Various quantities of crystalline ovalbumin were added to its antiserum to find the zone of dilution *in vitro* in which antigen and antibody occur in the supernatant fluids after the primary precipitin reaction. Serum from Rabbit 23 immunized to crystalline egg albumin, having a titer of 1:10,000. 5 per cent crystalline egg albumin in normal salt solution was added in diminishing amounts to undiluted Serum 23 according to the following table.

Tube.	Serum 23, undiluted.	5 per cent crystalline egg albumin.	Normal salt solution.	Precipitate after 18 hrs. in ice box.	Supernatant fluids.	
					For antigen.	For precipitin.
					0.2 cc. of supernatant fluid plus 0.2 cc. of Serum 23.	0.2 cc. of supernatant fluid plus 0.2 cc. of crystal- line albumin, diluted 1:10.
	cc.	cc.	cc.			
1	0.5	0.05	0.25	++	+++	+
2	0.5	0.01	0.25	+++	++	+
3	0.5	0.005	0.25	+++	++	++
4	0.5	0.001	0.25	++++	+	++
5	0.5	0.00075	0.25	+++	=	++
6	0.5	0.0005	0.25	++	o	++
7	0.5	0.0001	0.25	+	o	+++
8	0.5	0.00005	0.25	=	o	+++
Controls.						
9	0.5		0.25	o	o	++++
10		0.5	0.25	o	+	o
11		0.5	0.25	o	+	o

The overlapping shaded areas in the above table show the zone in the supernatant fluids in which free antigen and antibody (crystalline egg albumin and its precipitin) definitely coexist.

## EXPERIMENT IV.

*Crystalline Egg Albumin as Antigen.*

This experiment was done to show coexistence of antigen and antibody in the circulation of an immune animal.

June 2, 1916. Serum from Rabbit 23, immunized to crystalline egg albumin, having a titer of 1:5,000 before the last injection of antigen. 10.45 a.m. 3 cc. of 5 per cent solution of crystalline egg albumin were injected intravenously into Rabbit 23. 11.30 a.m. Bled from carotid 15 cc. 3 p.m. Tests were carried out as follows.

Tube.	Serum 23A before injection.	Serum 23B after injection.	5 per cent solution of crystalline egg albumin.		Normal salt solu- tion.	Normal rabbit serum.	Precipi- tate after $\frac{1}{2}$ hr. at 37°C.
			Dilution.	Amount.			
	cc.	cc.		cc.	cc.	cc.	
For antigen in Se- rum 23 B.							
1	0.2	0.2					++++
For precipitin in Se- rum 23 B.							
2		0.2	Undilut- ed.	0.2			+++
3		0.2	1 : 10	0.2			++
4		0.2	1 : 100	0.2			o
Controls.							
5					0.2		o
6						0.2	o
7	0.2	0.2			0.2		o
8	0.2	0.2				0.2	o
9			Undilut- ed.	0.2	0.2		o
10			"	0.2		0.2	o
11					0.2	0.2	o

Tube 1 shows that the serum of Rabbit 23 contained antigen un-  
united with its antibody 45 minutes after an injection of crystalline  
egg albumin. Tubes 2, 3, and 4 show that during this time free  
precipitin was still present in the blood of the immune animal.

In several experiments conducted like this one, similar results were  
obtained, showing that the sera of rabbits immunized to egg albumin  
contain uncombined antigen and antibody in the circulation for ap-  
proximately 48 hours after the last injection of the antigen.

In many of the precipitin reactions with crystalline egg albumin  
the maximum of precipitation occurred only when the albumin so-  
lution was diluted from 500 to 1,000 times. This phase of the pre-  
cipitin reaction is commonly known as the prozone, and is most read-  
ily explained as a phenomenon of colloidal relationship. In this  
particular case it seemed desirable to investigate the inhibition of  
precipitation effected by the albumin preparation, to relate it, if  
possible, to the protective influence exerted by this emulsoid on a  
sensitive gold sol. To show this effect, Experiment V was per-  
formed.



## EXPERIMENT V.

*Protective Action of Egg Albumin.*

June 1, 1916. This experiment was done to show the protective action of a solution of crystalline egg albumin upon the precipitation of human serum by its specific antiserum. To the serum from Rabbit 24, having a titer of 1:10,000 against human serum, various quantities of a 5 per cent solution of crystalline egg albumin were mixed with human serum. After  $\frac{1}{2}$  hour, anti-human serum from Rabbit 24 was added to these mixtures, as follows. The gold number of this preparation of egg albumin was 8.

Tube.	Human serum.		Anti-human Serum 24.		5 per cent solution of crystalline egg albumin.	Normal salt solution.	Precipitate after $\frac{1}{2}$ hr. at 37°C.
	Dilution.	Amount.	Dilution.	Amount.			
1	Undiluted.	cc. 0.2	Undiluted.	cc. 0.2	cc.	cc. 0.2	++++
2	"	0.2	1:5	0.2		0.2	+++
3	1:100	0.2	1:5	0.2		0.2	++
4	1:1,000	0.2	1:5	0.2		0.2	+
5	1:5,000	0.2	1:5	0.2		0.2	+
6	1:10,000	0.2	1:5	0.2		0.2	o
7	Undiluted.	0.2		0.2	0.2		++++
8	"	0.2	1:5	0.2	0.2		++
9	1:100	0.2	1:5	0.2	0.2		++
10	1:100	0.1	1:5	0.1	0.2	0.2	±
11	1:100	0.1	1:5	0.1	0.4		o
Controls.							
12	Undiluted.	0.2				0.2	o
13	"	0.2			0.2		o
14				0.2	0.2		o
15				0.2		0.2	o

These tests (Tubes 7 to 11) show that the presence of sufficient egg albumin prevents the flocculation of human serum by its antiserum. This effect of the solution of egg albumin is in accord with its well known protective action on other colloids, particularly its protection of a sensitive gold sol. against precipitation by electrolytes. The protective action in the above tests is obviously stronger than that exerted by the sera and may explain the long prozones commonly found in precipitin reactions with solutions of crystalline egg albumin.

The hypothesis of von Dungern<sup>8</sup> is insufficient to explain the fact that a serum containing both antigen and antibody, though clear at first, undergoes spontaneous precipitation upon long standing.<sup>1</sup> As

this precipitation progresses the amount of antigen and antibody gradually diminishes. This phenomenon is a matter of common observation when the sera of animals immunized to a mixture of protein are studied at successive intervals after an injection of the proteins used as antigen. Since it was shown by Experiments I and IV that a simple antigen such as edestin or crystalline egg albumin remains ununited with its homologous precipitin in the circulation of an immune rabbit for at least 24 hours after the last injection of the protein, a serum was readily obtained in which the slow spontaneous union of a single antigen with its antibody could be observed. This series of tests was carried out as follows.

## EXPERIMENT VI.

*Spontaneous Precipitation in a Serum Containing Antigen and Antibody (Crystalline Egg Albumin and Its Precipitin).*

July 27, 1916. Rabbit 25 immunized to crystalline egg albumin, having a serum with a titer of 1:25,000, was injected intravenously with 3 cc. of a 5 per cent solution of crystalline egg albumin. 1 hour later blood was drawn aseptically into sterile tubes. Serum obtained from this was divided into several lots and stored in sterile tubes in the ice box. Titrations for antigen and antibody were made as follows.

Tube.	Serum 25 after injection.	Serum 25 be- fore in- jection.	5 per cent solution of crystalline egg albumin.		Normal salt solu- tion.	Normal rabbit serum.	Precipitate.	
			Dilution.	Amount.			After $\frac{1}{2}$ hr. at 37°C.	After 24 hrs. in ice box.
	cc.	cc.		cc.	cc.	cc.		
For antigen.								
1	0.2	0.2					+++	++++
For precipitin.								
2	0.2		1:10	0.2			++	++
3	0.2		1:100	0.2			+	+
4	0.2		1:500	0.2			+	+
5	0.2		1:1,000	0.2			+	+
6	0.2		1:5,000	0.2			+	+
7	0.2		1:10,000	0.2			o	=
Controls.								
8	0.2				0.2		o	Slight precip- itate (spon- taneous).
9			1:10	0.2		0.2	o	o
10			1:10	0.2	0.2		o	o
11	0.2					0.2	o	o
12		0.2				0.2	o	o
13		0.2	1:25,000	0.2			+	+

Tube 1 shows that the serum contained a considerable amount of antigen, while Tubes 6 and 7 show that the precipitin titer of the same serum equalled 1:10,000. Tubes 8 to 13 were used to show that no non-specific precipitation occurred in any of the components of the primary reaction. With the subsequent tests, this series of controls was repeated invariably. Their results were always as anticipated and they will be omitted in detail from the following protocols.

The batches of Serum 25, kept in the ice box, showed definite spontaneous precipitation at the end of 24 hours.

July 28. Titration of the antigen and antibody content of this serum was carried out as follows.

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After $\frac{1}{2}$ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	cc.		cc.		
For antigen.							
1	Undiluted.	0.1	0.1			++	++++
2	1 : 10	0.1	0.1			+	+++
3	1 : 100	0.1	0.1			+	++
4	1 : 250	0.1	0.1			+	+
5	1 : 500	0.1	0.1			o	=
6	1 : 1,000	0.1	0.1			o	o
For precipitin.							
7	Undiluted.	0.1		1 : 1,000	0.1	+	+
8	"	0.1		1 : 5,000	0.1	=	=
9	"	0.1		1 : 10,000	0.1	o	o

This series of tests shows that the quantity of antigen present in Serum 25 (after injection) was sufficient to give a visible reaction when diluted 500 times. At the same time the precipitin titer of this serum containing free antigen was 1:1,000. In the previous 24 hours, however, while spontaneous precipitation had occurred in the serum, the precipitin titer decreased from 1:5,000 to 1:1,000.

Spontaneous precipitation continued, and at the end of 48 hours the following proportions of egg albumin and its precipitin were found to be present.

July 29. Serum 25 titrated as follows:

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After $\frac{1}{2}$ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	cc.		cc.		
For antigen.							
1	Undiluted.	0.1	0.1			++	+++
2	1 : 10	0.1	0.1			+	++
3	1 : 100	0.1	0.1			=	+
4	1 : 250	0.1	0.1			o	+
5	1 : 500	0.1	0.1			o	o
For precipitin.							
6	Undiluted.	0.1		1 : 500	0.1	+	+
7	"	0.1		1 : 1,000	0.1	o	o
8	"	0.1		1 : 5,000	0.1	o	o

July 31. 96 hours after the serum had been obtained, the tubes were turbid with considerable flocculated material in the sediment. The turbidity disappeared on warming the serum, but the sediment remained, giving evidence of the specific nature of this spontaneous precipitate. Titration of the serum at the end of 96 hours gave the following values for its content of free antigen and antibody.

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After $\frac{1}{2}$ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	cc.		cc.		
For antigen.							
1	Undiluted.	0.1	0.1			+++	+++
2	1 : 10	0.1	0.1			++	++
3	1 : 150	0.1	0.1			+	+
4	1 : 200	0.1	0.1			+	+
5	1 : 300	0.1	0.1			o	o
For precipitin.							
6	Undiluted.	0.1		1 : 100	0.1	=	+
7	"	0.1		1 : 350	0.1	o	o

This shows that during the course of spontaneous precipitation in the serum, both egg albumin and its precipitin became decreased. At the end of 96 hours the antigen titer had fallen to 1 : 200 and the precipitin titer to 1 : 100.

Subsequent titrations were carried out to follow the decrease of these anti-substances coincident with progress of spontaneous precipitation in their serum.

At the end of 144 hours the serum no longer contained precipitin, while the antigen titer had decreased to 1 : 100.

At the end of 196 hours the precipitin was still zero, while the antigen titer remained constant at 1 : 100.

The results of these titrations of the supernatant serum during spontaneous precipitation are summarized in Table II, and represented graphically in the curve of the reaction (Text-fig. 1).

TABLE II.

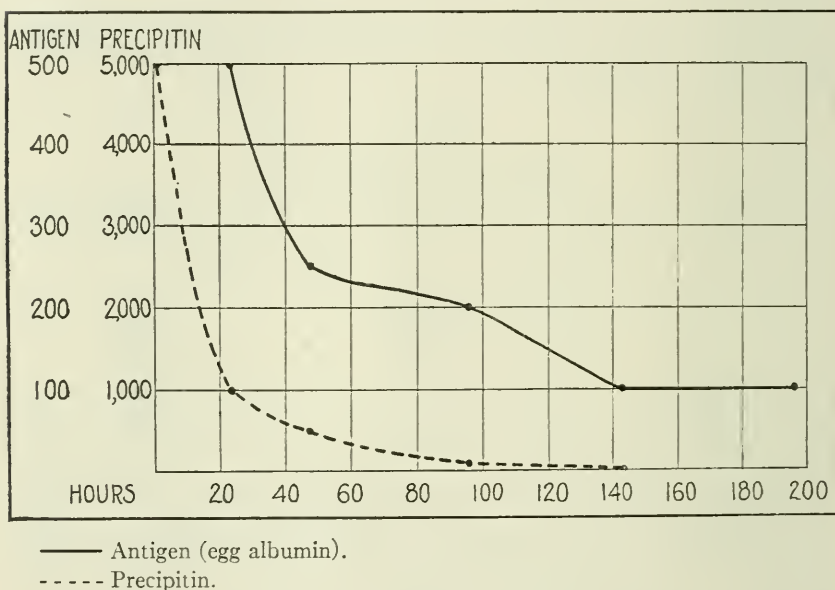
*Summary of Experiment VI.*

Hours.	Precipitin.	Antigen.
2	1 : 5,000	?
24	1 : 1,000	1 : 500
48	1 : 500	1 : 250
96	1 : 100	1 : 200
144	0	1 : 100
196	0	1 : 100

The chart of the observed amounts of antigen and antibody remaining dissociated in the supernatant fluid of the precipitin reaction suggests at once that the process has taken place according to a definite law. As the amounts of antigen and precipitin are stated only in terms of the dilution of mixtures whose protein content was not accurately estimated it is not possible to analyze this curve in detail. Experiments are being undertaken, therefore, to determine quantitatively the mutual relationship of the factors of this reaction. While, however, calculations are not possible which would reveal the nature of this reaction, it is permissible to point out that the curve is not unlike that of some colloidal precipitations. In this case, the amount of precipitate together with the amount of precipitin remaining in solution in the presence of the precipitable substance is apparently a function of the concentration of the precipitable substance; *e.g.*, crystalline egg albumin, the colloidal properties of which are indisputable. Although the experiment was not conducted upon a quantitative basis, the regularity of the curve of its results is striking. From this feature it is a fair assumption to suppose that the reacting



antibodies were strictly homologous, and that the absence of irregularity in the curve is evidence that the antigen was a relatively simple substance. This confirms the chemical evidence of the purity of the preparation of crystalline egg albumin used as antigen.



TEXT-FIG. 1. Graphic representation of the results of Experiment VI, showing the changes in the quantities of antigen and antibody during spontaneous precipitation in a serum containing these substances. The quantities are expressed by titration values.

#### SUMMARY.

1. In these studies several phases of the precipitin reactions were investigated by the use of purified proteins as antigens. These preparations were edestin from hemp-seed and crystalline ovalbumin from fresh eggs. The ovalbumin, isolated by the method of Hopkins and Pinkus, was apparently as pure as is obtainable by chemical means. This albumin, however, produced moderately severe anaphylactic reactions in animals sensitized with ovoglobulin. Anaphylactic tests of the individuality of a protein cannot be any longer regarded as the criterion of the purity of the substance as an antigen.

Wells and Osborne<sup>16</sup> have shown that proteins of considerable chemical difference may have a common antigenic group which causes mutual anaphylactic reactions in animals sensitized to these proteins. In particular, as egg globulin is a mixture of proteins, one of which is undoubtedly egg albumin, anaphylaxis produced by injections of albumin into animals sensitized to the so called globulin offers no evidence for or against the purity of the albumin. The character of the curves shown in Text-fig. 1 confirms the assumption, based upon chemical data, that crystalline egg albumin is a single protein.

2. With edestin and crystalline egg albumin as antigens, phases in the precipitin reaction were found in which these substances and their specific precipitins could be demonstrated to be coexistent but ununited in the same serum.

3. When edestin or crystalline egg albumin is injected into a rabbit immunized thereto, the antigen may be found in the circulating blood during 48 hours after its injection, while at the same time the animal maintains a high titer of free precipitin in its blood.

4. When the pure protein antigen is mixed in proper proportions with the serum of a specifically immunized rabbit and the resulting precipitate removed by centrifugation, the supernatant fluid contains both antigen and antibody.

5. The serum drawn from a rabbit during the period in which free antigen and antibody are coexistent in the circulation undergoes slow spontaneous precipitation when kept in sterile tubes in the ice box. The curve of this reaction is reproduced as Text-fig. 1. The relationships of the parabola indicate that the interaction of antigen and antibody takes place according to a definite law. When sufficient quantitative data are obtained to allow an analysis of this curve, the formulas for this reaction will undoubtedly throw light upon the chemical or physical nature of the process.

6. The protective action of the solution of egg albumin as a third colloid preventing precipitation in a reaction between human serum and its antibody was readily demonstrated. This observation and the constancy of the long prozone in precipitin test with egg albumin are in accord with the protective action of ovalbumin upon colloidal gold.



# THE FOCAL PULMONARY TUBERCULOSIS OF CHILDREN AND ADULTS.

BY EUGENE L. OPIE, M.D.

*(From the Pathological Laboratory of Washington University Medical School, St. Louis.)*

PLATES 68 TO 71.

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In order to obtain data concerning the relation of phthisis to tuberculous infection during childhood it has been desirable to determine the local frequency of infection among both children and adults. In a city such as St. Louis conditions of living differ widely from those in cities of the old world where the incidence of tuberculosis has heretofore been studied.

Statistics which have been obtained would be of little value if they had not demonstrated that here as in European cities tuberculous infection is well nigh universal among those who have reached adult life. A detailed examination of the localization and character of the lesions found in the lungs and elsewhere has furnished new data concerning tuberculous infection, for it has demonstrated that the healed or healing tuberculous lesions demonstrable in the lungs of many children and of nearly all adults have characters which serve to distinguish them from the progressive pulmonary tuberculosis of adults. They are identical with the tuberculosis of infancy and early childhood and exhibit little resemblance to the phthisis of adults.

The use of x-ray plates made from the lungs has proven an efficient means of determining the presence of healed tuberculous lesions within the substance of the organs. Since calcium salts are impervious to the x-rays very small calcified nodules may be readily identified in the plate (Figs. 1 to 9). Partially calcified foci containing caseous material of soft friable consistence are conspicuous in x-ray plates. Search for tuberculous lesions has been made in the lung hardened in formalin and, as far as necessary, cut into thin sections. In no in-

stance has the x-ray shadow been used as evidence of the presence of a calcified nodule. The difficulty of finding a nodule well depicted in the plate has often been great (Fig. 8), and in a number of instances no lesions would have been demonstrable without the aid of the plate. In one specimen a small intrapulmonary nodule obvious in the plate was long sought by careful palpation of sections from 0.5 to 1 cm. in thickness, and finally found within the angle at the bifurcation of a bronchus of medium size.

Lesions characterized by the presence of nodules which had undergone caseation were regarded as tuberculous. A calcified nodule surrounded by a fibrous capsule was regarded as a tuberculous lesion in which calcium had been deposited in caseous material. With few exceptions calcified nodules were found both within the substance of the lung and in regional lymphatic nodes at the hilus of that lobe in which the pulmonary lesion occurred. Although it is well known that tuberculous lesions in healing may form fibrous scars, fibrous nodules were not classified as tuberculous even when they formed wedge-shaped masses penetrating from the pleura into the substance of the apices. Wherever there was doubt concerning the nature of a lesion microscopic examination was made. In two instances tubercles were found by microscopic examination although none were recognized by the naked eye.

Pulmonary tuberculosis in infancy and early childhood is accompanied by enlargement and caseation of intrapulmonary bronchial and of tracheal lymph nodes. The pulmonary lesion may be situated in any part of the lungs and is not more frequently localized in the apices than elsewhere; there is indeed a tendency for the lesion to occupy the mid-part of either lung, frequently affecting the middle lobe on the right side or the corresponding part of the upper lobe on the left. There is in infancy little formation of fibrous tissue about the tuberculous lesion, but with increasing age encapsulation by fibrous tissue occurs more frequently. Cavities are much less frequently formed in children than in adults and usually occupy only a small part of the tuberculous lesion. Dissemination of tuberculosis frequently occurs and tuberculosis of the meninges, spleen, or other tissue is frequently associated with pulmonary tuberculosis and tuberculosis of bronchial lymphatic nodes.



Pulmonary tuberculosis of adults almost invariably affects the apices of the lungs. The disease tends to pursue a chronic course; there is often abundant formation of fibrous tissue and cavities are commonly present when the lesion is advanced. The disease does not implicate the regional lymphatic nodes at the hilus of the diseased lung. In the absence of acute miliary tuberculosis generalization with tuberculosis of the meninges, spleen, or other organs is uncommon unless the disease is approaching a fatal termination.

For the purpose of the present investigation the pulmonary tuberculous lesions which occur in childhood have been compared with those of adults. The age of 18 years has been arbitrarily selected as the line of division between adolescence and adult life. During the period within which autopsies were made upon fifty adults ninety-three children were examined. Tuberculosis was the cause of death in eleven children and in three adults; in the bodies of those who had died of other diseases, tuberculosis was found eleven times in children and forty-seven times in adults.

The incidence of tuberculosis at different ages is shown in Table I.

TABLE I.

Age.	No. of autopsies.	Tuberculosis.				In those who have died with other diseases.
		Present.	Fatal.	Non-fatal.		
Children.						
<i>yrs.</i>			<i>per cent</i>			<i>per cent</i>
Under 1	43	4	9.3	4	0	0
1- 2	16	1	6.2	1	0	0
2- 5	14	6	42.8	3	3	27.3
5-10	11	5	45.5	2	3	33.3
10-18	9	6	66.7	1	5	62.5
Adults.						
18-30	6	6	100	1	5	100
30-50	23	23	100	1	22	100
50-70	15	15	100	1	14	100
70+	6	6	100	0	6	100

The proportion of instances of fatal tuberculosis in Table I is of little significance since it depends upon the frequency with which tuberculosis is admitted to the two hospitals from which autopsies were obtained. Since all diseases are admitted to the St. Louis Children's Hospital the figures for children represent approximately the frequency of the disease in this city. Recognized tuberculosis is not usually admitted to the Barnes Hospital. When fatal tuberculosis is excluded the figures representing the incidence of tuberculous infection among those who have died of other diseases are an index of the frequency of infection in the general population. In most instances the anatomical characters of the lesion indicate that it has long existed in the body and bears no relation to the fatal illness. Before the age of 2 years the disease is almost always fatal, but later there is a progressive increase in the number of infected individuals. Evidence of infection has been found in all adults.

The result of this incidental study of the occurrence of tuberculosis in a small number of autopsies is in accord with the studies of Naegeli made in Zurich and of Burkhardt made in Dresden upon a far larger number of autopsies. Among adults Naegeli<sup>1</sup> found tuberculosis in 97 per cent and Burkhardt<sup>2</sup> in 90 per cent of all individuals.

A survey of tuberculosis which has been found furnishes a satisfactory basis for comparison between the lesions of infants and adolescents on the one hand and of adults on the other. The well known characters which distinguish the tuberculosis of childhood will be cited briefly.

#### *Tuberculosis of Children.*

*Location and Character of the Pulmonary Lesions of Childhood.*—Tuberculosis of infancy has occurred five times and in all instances was fatal within the 1st or 2nd year of life (Text-fig. 1 and Fig. 1). Massive caseous lesions in some instances have implicated a considerable part of one lung but do not affect the apex more frequently than other parts. The middle lobe on the right side in one instance has been almost wholly caseous. Small cavities are occasionally formed. These lesions have exhibited no tendency to heal. After

<sup>1</sup> Naegeli, O., *Virchows Arch. path. Anat.*, 1900, clx, 426.

<sup>2</sup> Burkhardt, A., *Z. Hyg. u. Infektionskrankh.*, 1906, liii, 139.

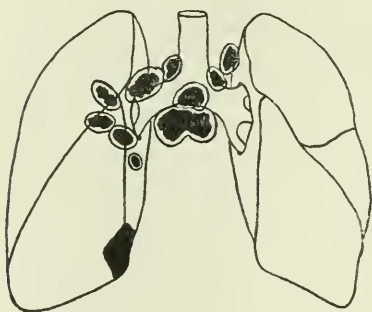
the end of the 2nd year tuberculous lesions have usually been small, seldom more than 1.5 cm. in diameter, and may be situated in any part of the lung substance (Text-figs. 2 to 6). They may be designated focal lesions and differ from the lesions of infancy only in size. A tendency to heal may be indicated by encapsulation of the caseous focus with fibrous tissue. In one instance an encapsulated caseous nodule has been found in a child only 2 years of age. The caseous material undergoes calcification and at first assumes a dry friable mortar-like consistency; later it is converted into material of stony hardness. Nodules consisting of a calcified center surrounded by a fibrous capsule and less than 1 cm. in diameter have been found in a considerable proportion of all children between the ages of 4 and 15 years (Figs. 2 and 3). More than two-thirds of all these tuberculous foci have been in contact with the pleural surface of the lung (Text-figs. 1, 4, and 6).

The study of Ghon<sup>3</sup> based upon a large number of observations has demonstrated the distribution of pulmonary tuberculous infection in children. The distribution of the lesion in the cases which I have studied is cited in order that the lesions of children and of adults may be compared. In most instances there has been only one focus of pulmonary tuberculosis (Text-figs. 1, 2, and 5); smaller nodules nearby may be obviously secondary to a larger lesion (Text-fig. 3). In three instances there have been two or more separate foci of infection (Text-fig. 6). The distribution of these lesions in the various lobes of the lung have been as follows: right upper lobe, 7; right middle lobe, 3; right lower lobe, 5; left upper lobe, 5; left lower lobe, 5. These figures are cited to show that the tuberculous lesions of childhood are impartially scattered in the substance of the lung. In one instance in a child 11 years of age tuberculosis had the usual characters of the phthisis of adults; the apices of both lungs were affected and cavities of considerable size occurred in both lungs. It is well known that fatal tuberculosis of this type is not uncommon after the 6th or 7th year.

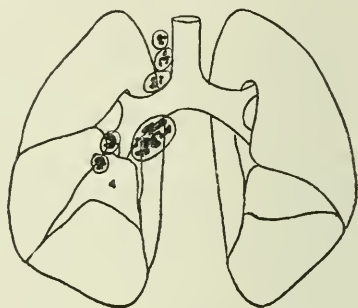
*Relation of the Pulmonary Focus to Lesions in Regional Lymphatic Nodes.*—The most conspicuous character of tuberculosis in children

<sup>3</sup> Ghon, A., *Der primäre Lungenherd bei der Tuberkulose der Kinder*, Berlin, 1912.

is the extent of involvement of regional lymphatic nodes. In nineteen instances caseous or calcified tuberculous lesions were found both in the substance of the lung and in the lymphatic nodes either



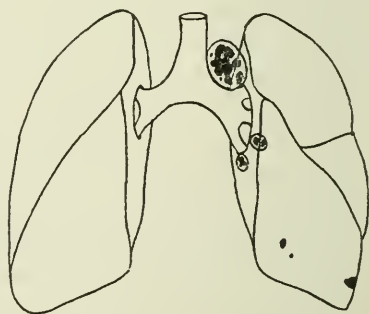
TEXT-FIG. 1. Posterior view of the lungs of a child, age 9 months, who died of pulmonary tuberculosis, meningitis, and general miliary tuberculosis. There is a caseous focus in the left lower lobe and tuberculosis of the regional lymphatic nodes. Compare with Fig. 1 showing the same lungs.



TEXT-FIG. 2. Median view of the lungs of a child, age 2½ years, who died of ileocolitis. There is a caseous focus in the right lower lobe and caseation of the regional lymphatic nodes.

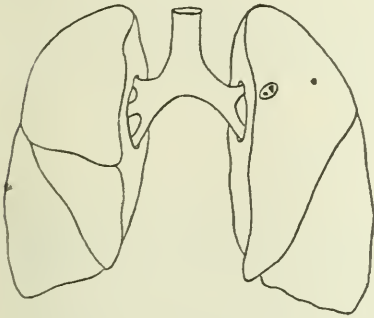


TEXT-FIG. 3. Anterior view of the lungs of a child, age 3 years, who died of postdiphtheritic paralysis and bronchopneumonia. There is a caseous focus surrounded by tubercles in the left upper lobe and caseation of the regional lymphatic nodes.

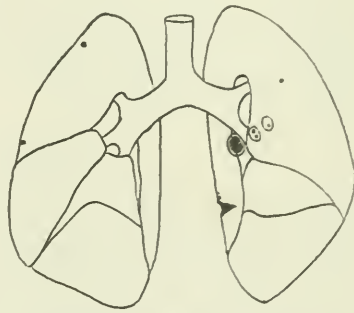


TEXT-FIG. 4. Posterior view of the lungs of a child, age 6 years, who died of otitis media and acute suppurative meningitis. There are three calcified nodules in the lower right lobe and in the regional lymphatic nodes. Compare with Fig. 2 from the same lungs.

within the lung near its hilus or in the adjacent peribronchial or tracheal nodes. In only one instance has a lesion been found in the lymphatic nodes although none was demonstrable in the substance of the lung. It is not improbable that small lesions may be overlooked. If caseation has failed to occur complete resolution of the lesion is possible. In one instance a tuberculous focus was found in the lung although none was found in the adjacent lymphatic nodes. In the child with apical tuberculosis mentioned above, the regional lymphatic nodes were enlarged and contained tubercles but were not caseous.



TEXT-FIG. 5. Anterior view of the lungs of a child, age 10 years, who died of osteomyelitis and pyemia. There is an encapsulated, caseous, and partly calcified nodule in the left upper lobe and a lymphatic node nearby contains encapsulated caseous nodules.



TEXT-FIG. 6. Median view of the lungs of a child, age 15 years, who died of an abscess of the brain and bronchopneumonia. There are calcified foci in the left upper and lower lobes and calcification of regional lymphatic nodes. In the right upper lobe there are encapsulated caseous foci but no caseation of the lymph nodes on this side. Compare with Fig. 3 from the same lungs.

Ghon, studying the tuberculosis of children, has shown the almost constant association of pulmonary lesions and lesions of the pulmonary and bronchial nodes. He has found that the pulmonary lesion often exhibits characters which indicate that it is older than the lesion of the adjacent lymphatic nodes and this observation he believes furnishes evidence that the pulmonary lesion is primary and the lymphatic foci are secondary. In several instances the specimens which



I have examined have exhibited a similar relation between pulmonary and lymphatic lesions; the lesion of the lung has been encapsulated whereas there has been no encapsulation of the lymphatic lesion.

The susceptibility of the lymphatic nodes of infants and very young children to tuberculosis is well shown by great enlargement and widespread caseation of the affected nodes. Masses of greatly enlarged almost wholly caseous lymphatic nodes occupy the hilus of the lung in which the pulmonary focus is situated, and similar masses fill the spaces between trachea and lung or the angle at the bifurcation of the trachea (Text-figs. 1 to 4). At a more advanced age there is less enlargement and caseation of regional lymphatic nodes and in children between the ages of 10 and 18 years it is not uncommon to find small caseous or calcified nodules in only one or two lymphatic nodes (Text-figs. 5 and 6).

#### *Tuberculosis of Adults.*

Table I shows that tuberculosis has been found in all of fifty individuals above the age of 18 years. The criteria previously described have been kept in mind and no lesions regarded as doubtful have been recorded as tuberculous. Tuberculous lesions characterized by the presence of fresh tubercles have been designated active tuberculosis. Lesions in which caseous foci are completely surrounded by fibrous tissue have been found much more frequently; in this group are included a considerable number of lesions in which the caseous material has a dry mortar-like consistence, is obviously partially calcified, and gives a conspicuous shadow upon the x-ray plate. Lesions in which a firmly calcified center is surrounded by a capsule of fibrous tissue have been classified as healed tuberculosis. Table II shows the frequency of active, encapsulated, and healed tuberculosis in the series of autopsies which have been studied. When active and encapsulated lesions occurred in the same individual the process has been listed as active; when encapsulated and calcified lesions occurred together the disease was classified as encapsulated. Tuberculosis was regarded as healed only when all the lesions were firmly calcified and surrounded by fibrous tissue.

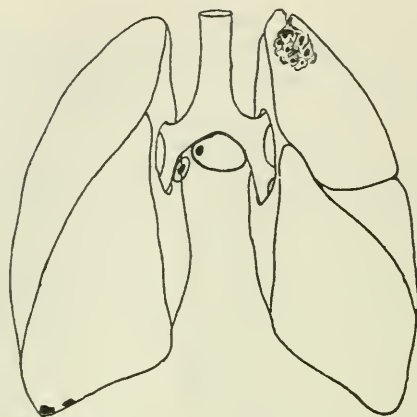
TABLE II.

Age.	No. of autopsies.	Active tuberculosis.	Encapsulated caseous lesions.		Healed tuberculosis.		
Children.							
<i>yrs.</i>			<i>per cent</i>		<i>per cent</i>		<i>per cent</i>
Under 1	43	4	9.3	0	0	0	0
1- 2	16	1	6.2	0	0	0	0
2- 5	14	5	35.7	1	7.1	0	0
5-10	11	2	18.2	1	9.1	2	18.2
10-18	9	1	11.1	4	44.4	1	11.1
Adults.							
18-30	6	1	16.7	3	50.0	2	33.3
30-50	23	2	8.7	12	52.2	9	39.1
50-70	15	2	13.3	7	46.7	6	40.0
70+	6	1	16.6	3	50.0	2	33.3

The table shows that active tuberculosis is very frequent between the ages of 2 and 10 years. These lesions are often of small size and undergo encapsulation. Latent or encapsulated tuberculosis is found in approximately half of all individuals over 10 years of age. Many of these lesions heal completely and a third of all adults have firmly calcified foci which represent former tuberculous infection.

The frequency of healed tuberculosis in the autopsies which I have studied is less than that found by Naegeli and by Burkhardt, and the proportion of latent encapsulated tuberculosis is greater. I have not recorded the pulmonary lesion as healed unless it has been compactly calcified and have excluded from the group of healed lesions cases in which caseous tuberculosis was found in regional lymphatic nodes.

Tuberculous lesions of the lungs may be separated into two sharply definable groups: (1) Lesions occupying the apices of the lungs, tending to spread diffusely through the tissue of the apex and unaccompanied by caseation (or calcification) of the regional lymphatic nodes represent what is generally regarded as the usual type of tuberculosis of adults (Text-figs. 7 and 8). This lesion which is often fatal may undergo complete healing and in a considerable proportion of autopsies upon individuals who have presented no evidence of



TEXT-FIG. 7. Posterior view of the lungs of a negro, age 39 years, who died of aneurysm of the aorta. There are two calcified nodules in the lower left lobe and similar nodules in the regional lymphatic nodes. There is a diffuse tuberculous lesion with caseation and fibrosis at the apex of the right lung. There is no caseation of the lymphatic nodes on the right side.



TEXT-FIG. 8. Median view of the lungs of a white man, age 74 years, who died of general arteriosclerosis, chronic diffuse nephritis, and fibrous myocarditis. There are calcified nodules in the right lower lobe and calcified and caseous foci in the regional lymphatic nodes. Near the apex of the left upper lobe is a diffuse tuberculous lesion with caseation and fibrosis. There is no caseation of the lymphatic nodes on the left side.

pulmonary tuberculosis the apex of one or both lungs is occupied by an area of diffuse fibrous induration within which occur caseous or calcified nodules. For convenience I shall designate this lesion apical tuberculosis. (2) The second group consists of circumscribed lesions situated in any part of the lung, not more frequently in the upper parts of the upper lobes than elsewhere, and accompanied by similar caseous or calcified lesions of the regional lymphatic nodes (Text-figs. 9 to 20). In the lungs of adults who have died with disease other than tuberculosis this is the commonest type of tuberculosis. These lesions have the distribution and characters of the circumscribed tuberculous lesions which begin to appear after the 2nd year of life and during later childhood are often found to be encapsulated and even calcified. They may be designated focal tuberculosis.

The relative frequency of the two types of pulmonary tuberculosis in the present series of autopsies is shown in Table III.

TABLE III.

Age.	No. of autopsies.	Focal pulmonary tuberculosis.	Apical tuberculosis.		
Children.					
<i>yrs.</i>			<i>per cent</i>		<i>per cent</i>
Under 1	43	4	9.3	0	0
1- 2	16	1	6.2	0	0
2- 5	14	6	42.8	0	0
5-10	11	5	45.5	0	0
10-18	9	5	55.5	1	11.1
Adults.					
18-30	6	5	83.3	1	16.7
30-50	23	21	91.3	3	13.0
50-70	15	14	93.3	4	26.7
70+	6	6	100.0	3	50.0

It is noteworthy that the incidence of focal tuberculosis increases continuously after the 2nd year of life. Comparison with Table I shows that the focal type of tuberculosis is frequently fatal in early childhood whereas after the 10th year it is rarely a cause of death. At the age of 18 a large proportion of all individuals have acquired these focal lesions but a few have escaped; at least 92 per cent of all

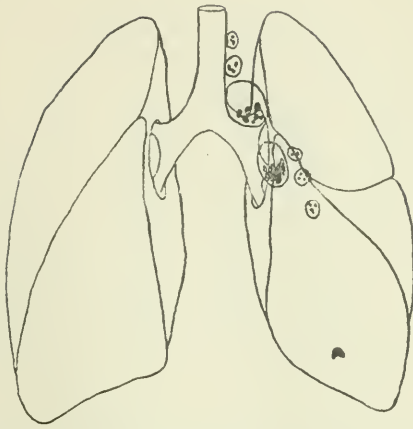
adults possess focal lesions. Apical phthisis in the present series of autopsies makes its appearance at the age of 11 years and subsequently apical lesions increase in frequency from adolescence to old age. The existence of focal lesions on the one hand does not preclude the occurrence of apical tuberculosis. On the other hand, in one instance of apical tuberculosis between the 18th and 30th years there was no focal lesion and apical tuberculosis was associated with caseous tuberculosis of the regional lymphatic nodes and general miliary tuberculosis. I shall discuss the relation of focal to apical tuberculosis in a subsequent paper.

*Location and Characters of the Focal Tuberculosis of Adults.*—The circumscribed tuberculous foci which occur in the lungs of adults do not differ in character and distribution from those which are found in older children. They rarely exceed 1 cm. in diameter and the smaller nodules perhaps not more than 1 mm. across are found with much difficulty even when they are calcified and cast a conspicuous shadow upon the x-ray plate. Attention is often first attracted to these pulmonary nodules by the more conspicuous lesions almost always present in the regional lymphatic nodes.

In the greater number of lungs there is only one focus of tuberculous infection (Text-figs. 9 to 12); when small nodules are near a relatively large focus they have been regarded as secondary to it (Text-figs. 13 and 14). In about a third of the autopsies studied two foci of pulmonary infection have been present (Text-figs. 15 to 18), and in several instances three (Text-fig. 19), four, or more foci have been found. In one instance (Text-fig. 20 and Fig. 9) in the absence of any evidence of healed miliary tuberculosis in other organs a great number of firmly calcified nodules varying in size were sprinkled throughout the lungs so that every lobe contained numerous foci of infection.

The distribution of focal tuberculous lesions in the lobes of the lung has been as follows: right upper lobe 17; right middle lobe 5; right lower lobe 15; left upper lobe 14; left lower lobe 15. The distribution of these lesions in the adult lung is almost identical with that in the lungs of children and occurs in the different lobes in approximate proportion to their volume. Nearly one-half of these focal lesions are situated immediately below the pleural surface (Text-

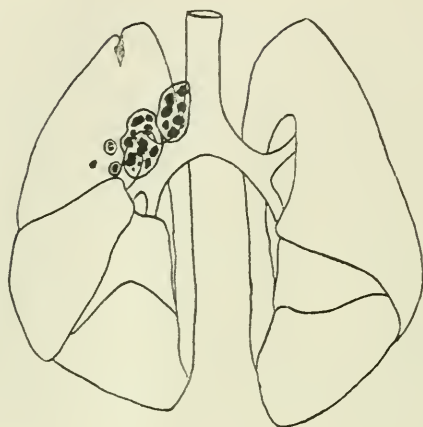




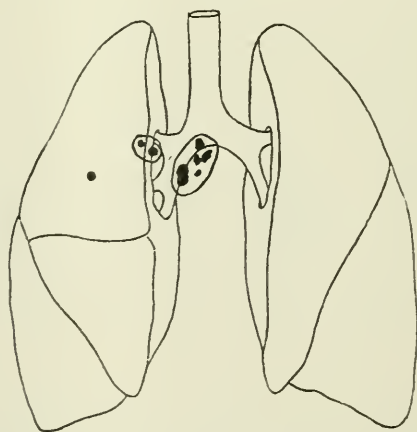
TEXT-FIG. 9. Posterior view of the lungs of a white man, age 19 years, who died of acromegaly, adenoma of the hypophysis, and bronchopneumonia. There is an encapsulated caseous focus in the right lower lobe and similar nodules in the regional lymphatic nodes.



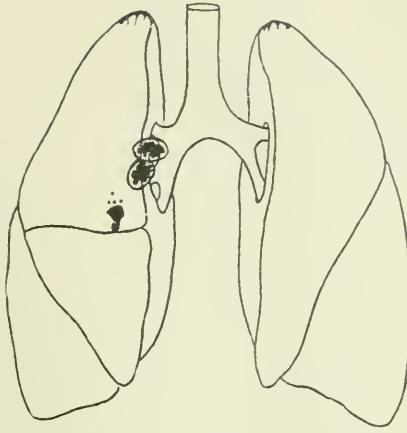
TEXT-FIG. 10. Median view of the lungs of a white man, age 35 years, who died of Hodgkin's disease, chronic diffuse nephritis, and cirrhosis of the liver. There are encapsulated, partially calcified caseous foci in the lower right lobe and in adjacent lymphatic nodes.



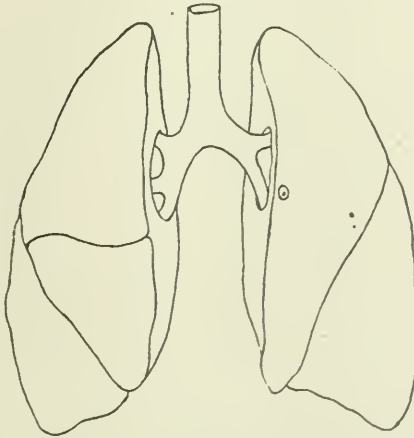
TEXT-FIG. 11. Median view of the lungs of a white woman, age 18 years, who died of typhoid fever. There are encapsulated, partially calcified, caseous foci in the right upper lobe and in adjacent lymphatic nodes.



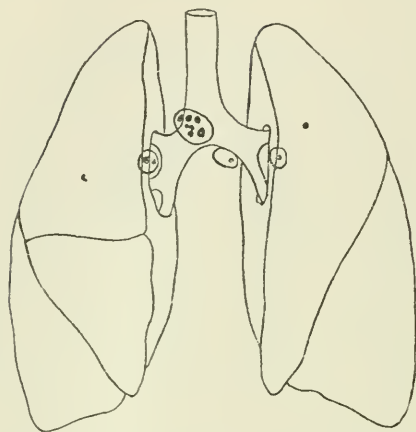
TEXT-FIG. 12. Anterior view of the lungs of a white woman, age 30 years, who died of Hodgkin's disease. There are encapsulated nodules in the right upper lobe and in adjacent lymphatic nodes.



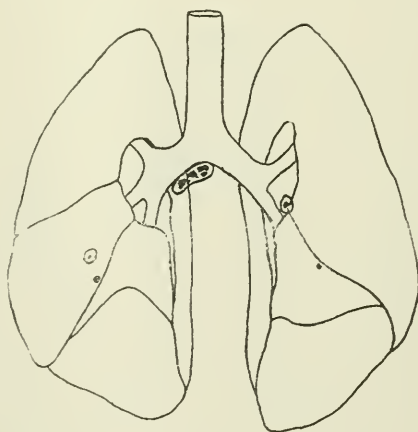
TEXT-FIG. 13. Anterior view of the lungs of a white woman, age 34 years, who died of Addison's disease, and tuberculosis of the adrenal glands, spleen, and left Fallopian tube. There are encapsulated, partially calcified, caseous nodules in the right upper lobe and in adjacent lymphatic nodes. These lesions appear to be older than those of the adrenal and spleen, etc. Compare with Fig. 4.



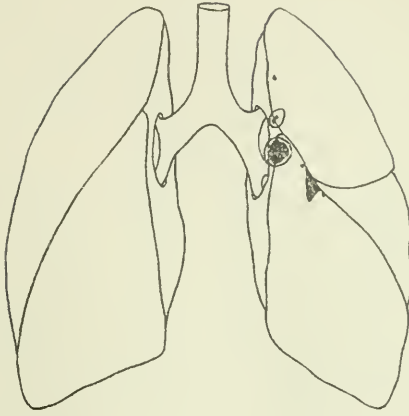
TEXT-FIG. 14. Anterior view of the lungs of a white man, age 56 years, who died of general arteriosclerosis, small granular kidneys, and hypertrophy of the heart. Inconspicuous calcified nodules occur in the left upper lobe and in a lymphatic node. Compare with Fig. 7.



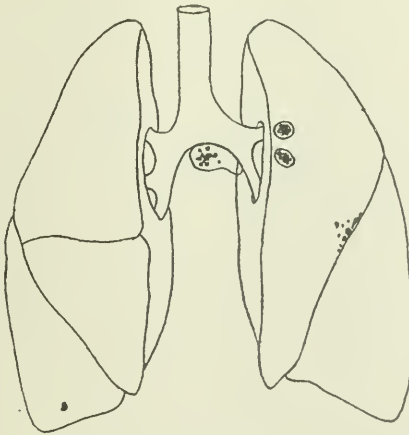
TEXT-FIG. 15. Anterior view of the lungs of a white man, age 71 years, who died of general arteriosclerosis, small granular kidneys, and hypertrophy and dilatation of the heart. There are two calcified nodules in the lungs and calcified and encapsulated caseous lesions in the lymphatic nodes.



TEXT-FIG. 16. Median view of the lungs of a white woman, age 48 years, who died following an operation for carcinoma of the cervix uteri. There are calcified and encapsulated caseous nodules in the lungs and in the lymphatic nodes. Compare with Fig. 5.

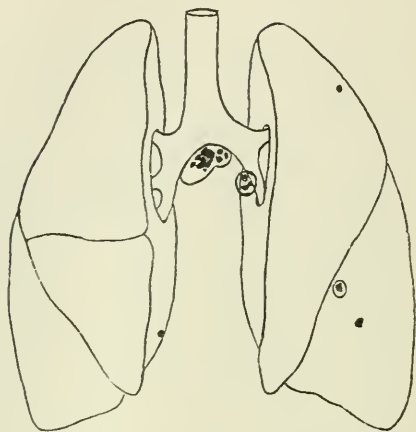


TEXT-FIG. 17. Posterior view of the lungs of a white man, age 80 years, who died of cirrhosis of the liver and chronic endocarditis. There is a calcified mass below the pleura of the right lower lobe and small calcified nodules occur in the pleura nearby. There is a calcified nodule in the right upper lobe and the lymphatic nodes at the hilus of the right lung are partially calcified.

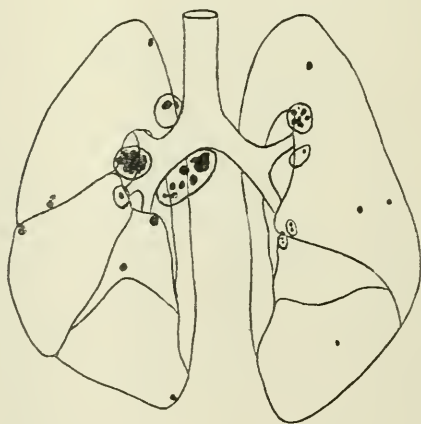


TEXT-FIG. 18. Anterior view of the lungs of a white man, age 46 years, who died of *æstivo-autumnal malaria*. There is a group of calcified nodules in contact with the interlobular pleura of the left upper lobe. There is a calcified nodule in the right lower lobe. There are partially calcified lymphatic nodes at the hilus of the left lung but none on the right side. Compare with Fig. 6 from the same lungs.





TEXT-FIG. 19. Anterior view of the lungs of a white woman, age 50 years, who died of small granular kidneys, arteriosclerosis, and dysentery. There are multiple calcified nodules in the lungs and similar lesions in the lymphatic nodes. Compare with Fig. 8 from the same lungs.



TEXT-FIG. 20. Median view of the lungs of a negress, age 36 years, who died following panhysterectomy for myomata of the uterus. There are multiple calcified nodules in all the lobes and numerous partially calcified lymphatic nodes. Compare with Fig. 9 from the same lungs. The text-figure shows only those nodules which were identified in the lung. The x-ray plate shows the presence of a far larger number of calcified nodules.

figs. 13, 17, 18, 20, etc.). It is not uncommon to find a calcified nodule immediately below the puckered pleura and about it upon the adjacent pleura a group of small nodules (Text-figs. 17 and 18 and Fig. 6). Fibrous adhesions usually bind together the adjacent pleural surfaces.

In children freshly caseous focal lesions are predominant, whereas in adults encapsulated or calcified nodules are much more common. The following classification serves to indicate the character of the focal lesions which were found in adults: freshly caseous, 1; caseous and encapsulated, 5; caseous and encapsulated with beginning deposit of calcium salts producing dry chalky or mortar-like material, 12; firmly calcified, 48. In two instances tuberculous foci were found in the substance of the lung but none were found in lymphatic nodes.

*Relation of the Pulmonary Tuberculous Focus to Tuberculosis of the Regional Lymphatic Nodes.*—When a localized focus of active or pre-existing tuberculous infection has been found in one of the lobes of the lung a similar lesion, with few exceptions, has been found in intrapulmonary lymphatic nodes nearby or in intra- or extrapulmonary nodes near the hilus of the lobe affected (Text-figs. 9 to 20). In instances in which pulmonary lesions occurred in more than one lobe tuberculous lesions have been usually found in regional lymphatic nodes corresponding to each of the affected lobes (Text-figs. 15 to 17); but occasionally with multiple lesions some were unaccompanied by lesions of the adjacent lymphatic nodes (Text-figs. 18 and 19). The lesion of the lymphatic node is usually larger and more readily found than the pulmonary lesion and frequently there is a chain of tuberculous nodes beginning in the lung and extending along the corresponding bronchus to one or the other side of the trachea (Text-figs. 9 and 11). In correspondence with the distribution of tuberculosis often seen in childhood a large mass of nodes containing calcified nodules is found in adults at the bifurcation of the trachea and in the spaces above the bronchi between the trachea and lung (Text-figs. 9, 11, 12, 18, 19, and 20). Text-figs. 7 to 20 and Figs. 4 to 9 indicate the size and distribution of the lesions of the lymphatic nodes and their position in relation to the pulmonary lesion. In most instances irregular calcified masses occur within indurated lymphatic nodes deeply pigmented with carbon particles. In other instances

spherical nodules 2 or 3 mm. in diameter consisting of a caseous or partially calcified mortar-like center and a thick fibrous capsule are scattered within the substance of enlarged pigmented lymphatic nodes. Freshly caseous lesions are rarely seen in adults. The lesion of the regional lymphatic node is usually similar to that within the lung, both being either caseous or calcified, but in eight instances the pulmonary focus was firmly calcified whereas the lymphatic lesion was caseous. In two instances this relation was reversed.

Of the tuberculous lesions which have occurred in the lymphatic nodes and are unaccompanied by lesions of the lung two consist of typical gray fibroid tubercles identified by microscopic examination and three are calcified nodules. In one specimen a large lymphatic node at the bifurcation of the trachea contains firmly calcified foci: in a second a minute calcified nodule is found with the aid of an x-ray plate in a lymphatic node near the hilus of the left upper lobe and a minute shadow of similar size occurs in the lung substance nearby, but careful examination has failed to reveal a pulmonary lesion. In a third instance a calcified nodule surrounded by a fibrous capsule 4 mm. in diameter occurs in a lymphatic node below the bifurcation of the trachea and a second similar nodule occurs in a lymph node above the left bronchus; a pearly white nodule 2 mm. in diameter is found in the substance of the left lower lobe but there is no evidence of caseation or of calcification. The characters of these lesions suggest no doubt that they are identical with similar lesions found in association with tuberculous foci in the lung substance.

The foregoing observations have shown that tuberculous infection is practically universal. Dissemination of the disease among adults is so widespread that readily recognizable tuberculous lesions have been found in all of fifty individuals above the age of 18 years. First infection in almost all of those who reach adult life occurs in childhood and has the characters of a first infection in animals since it tends to implicate regional lymphatic nodes. Koch showed that a second infection of an animal already tuberculous shows greater tendency to heal and does not extend to regional lymphatic nodes, and this observation has been confirmed by numerous observers. Apical tuberculosis usually exhibits the characters of a second infection, since it pursues a chronic course and is unaccompanied by tuberculosis of regional lymphatic nodes.

## CONCLUSIONS.

Evidence of tuberculous infection has been found in the lungs of all of fifty adults who have been examined. Approximately one-half of all adults have encapsulated lesions of the lungs or bronchial lymphatic nodes, whereas in one-third pulmonary and lymphatic lesions are firmly calcified and completely healed.

Tuberculous pulmonary lesions of adults who have died of diseases other than tuberculosis are of two types: (1) apical tuberculosis similar to the usual type of fatal phthisis and unaccompanied by caseation of the regional lymphatic nodes; (2) focal tuberculosis not more commonly situated in the apices of the lungs than elsewhere and accompanied by caseation (or calcification) of the adjacent lymphatic nodes.

Focal pulmonary tuberculosis of adults is identical with the tuberculosis of childhood. It occurs in at least 92 per cent of all adults. It may be acquired between the ages of 2 and 10 years but in more than half of all individuals (in this city) makes its appearance between the ages of 10 and 18 years.

Tuberculosis of children does not select the apices of the lungs, is accompanied by massive tuberculosis of regional lymphatic nodes, and exhibits the characters of tuberculosis in a freshly infected animal, whereas tuberculosis which occurs in the pulmonary apices of adults has the characters of a second infection. Almost all human beings are spontaneously "vaccinated" with tuberculosis before they reach adult life.

## EXPLANATION OF PLATES.

## PLATE 68.

FIG. 1. X-ray plate of the lungs of a child, age 9 months. The shadows correspond to caseous foci at the base of the lower left lobe and in the regional lymphatic nodes as shown in Text-fig. 1.

FIG. 2. X-ray plate of the lungs of a child, age 6 years, showing the calcified foci indicated in Text-fig. 4.

FIG. 3. X-ray plate of the left lung of a child, age 15 years, showing the calcified foci depicted in Text-fig. 6.

## PLATE 69.

FIG. 4. X-ray plate of the lungs of a white woman, age 34 years, who died of Addison's disease. The positions of the right and left lungs are reversed. Compare with Text-fig. 13 which shows the position of the tuberculous lesions indicated by shadows in the plate.

FIG. 5. X-ray plate of the lungs of a white woman, age 48 years. The positions of the right and left lungs are reversed. Compare with Text-fig. 16 from the same lungs.

## PLATE 70.

FIG. 6. X-ray plate of the lungs of a white man, age 46 years. The positions of the right and left lungs are reversed. Compare with Text-fig. 18 from the same lungs.

FIG. 7. X-ray plate of the lungs of a white man, age 56 years, showing small calcified nodules which were found in the lungs with much difficulty. Compare with Text-fig. 14 from the same lungs.

## PLATE 71.

FIG. 8. X-ray plate of the lungs of a white woman, age 50 years. The positions of the right and left lungs are reversed. Compare with Text-fig. 19 from the same lungs.

FIG. 9. X-ray plate of the lungs of a negress, age 36 years, showing a large number of calcified nodules in both lungs and in lymphatic nodes. The positions of the right and left lungs are reversed. Compare with Text-fig. 20 from the same lungs.





FIG. 1.



FIG. 2.



FIG. 3.

(Opie: Focal Pulmonary Tuberculosis.)





FIG. 4.



FIG. 5.

(Opie: Focal Pulmonary Tuberculosis.)





FIG. 6.



FIG. 7.

(Opie: Focal Pulmonary Tuberculosis.)







FIG. 8.



FIG. 9.

(Opie: Focal Pulmonary Tuberculosis.)



## A CLASSIFICATION OF NON-HEMOLYTIC STREPTOCOCCI.

BY RALPH A. KINSELLA, M.D., AND HOMER F. SWIFT, M.D.

(From the Medical Clinic of the Presbyterian Hospital, Columbia University, New York.)

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No group of bacteria has been suspected of etiologic relationship with so many and diversified disease processes as the streptococcus. At the same time there is no group which suffers more confusion in the relationship of its numerous members. The purpose of the present work was to attempt a classification of twenty-eight strains of non-hemolytic streptococci by a comparative study of their biochemical and immunological reactions.

Since the streptococcus was first recognized as an infectious agent by Fehleisen (1) in 1882, various methods of classifying these organisms have been proposed. The object of every attempt to classify has been to determine whether all streptococci are identical with accidental differences in pathogenicity and cultural features or whether the differences noted constitute more or less fixed characteristics, which demand separate grouping for such members. All the schemes can be included in those dealing with morphology, the ability to produce special lesions, and immunological and biochemical reactions.

Classification on the basis of morphology was abandoned early because of the influence which cultural environment was found to have on morphology. The terms *longus* (2), *brevis*, and *conglomeratus* (3) are now considered merely descriptive of growth phenomena and not of fundamental properties.

The opinion that certain streptococci have a definite etiologic relationship to certain diseases was natural, since they were frequently associated with these diseases. Thus Fehleisen (1) assumed a certain variety to be the cause of erysipelas, while Rosenbach (4) was the first to call attention to the importance of the variety which he called *pyogenes*, in the production of local purulent processes. Similarly, scarlet fever and smallpox were regarded as being of streptococcus origin. This supposition, however, was weakened by the failure to establish

experimental proof of their etiologic rôle in these diseases, and further, by the finding of streptococci in the throat, gastro-intestinal tract, and feces of normal individuals. It might be noted that while formerly a special streptococcus was thought to be etiologically responsible in a given disease, many modern believers in the unity of streptococci prefer to regard them all as identical and their capacity of producing any given disease as merely an accidental and temporary property.

The relation of streptococcus to disease as well as the relationship of different streptococci to one another was at first based upon the results of agglutination reactions. Van de Welde (5), the first to study this reaction, reported (6) a specific agglutination between a univalent serum and its homologous streptococcus. Baginsky and Sommerfeld (7) found in the serum of scarlet fever patients specific agglutinins for streptococci isolated from the throats of such patients. Similar findings were reported by Hasenknopf and Salge (8) and Moser and von Pirquet (9). De Waele and Sugg (10) isolated streptococci from the blood of cases of smallpox both before and after death and found that the sera of smallpox patients agglutinated the variety of streptococcus obtained, while sera from unvaccinated individuals failed to give positive reactions. Soon, however, discordant reports appeared. Meyer (11) tested ten streptococci against four monovalent sera obtained by immunizing rabbits with four of the strains, and found that cross agglutination occurred. Nevertheless, the organisms from purulent processes were not affected by sera immune to streptococci from the throats of scarlet fever and rheumatism patients. From these observations he concluded that agglutination reactions could be used to separate the streptococci of angina from those of septic processes. Other workers then began to report promiscuous cross agglutination between the various streptococci. Weaver (12), investigating the serum of scarlet fever patients, concluded that the agglutination reaction in this disease was in no way specific. Neufeld (13) thought that, in certain cases at least, agglutinability varied inversely with virulence. Since most of the strains that he used were capable of being made highly virulent for white mice it now seems probable that they were of the hemolytic variety. Aronson (14), investigating the value of serum in the treatment of so called streptococcus infections, concluded that a polyvalent serum was necessary on account of the failure of one streptococcus from a given disease to be agglutinated by a serum produced by immunizing with another streptococcus from the same disease. He thus argued against the constancy of streptococci as specific producers of disease. Marmorek (15) reviewed the investigations that had been made up to 1902 and concluded that all human pathogenic streptococci were the same. This view was later supported by Zoeppritz (16). Fischer (17) tested twenty-one streptococci from various pathological and normal sources against sera immune to eight representative members of the series and obtained such promiscuous cross agglutination that he decided a great multiplicity of strains existed, and that the agglutination reaction was not effective in grouping them.

From the foregoing it will be seen that the question of classification was unsettled and no plan for grouping streptococci had found wide acceptance. Two courses of procedure were open. One, the result of the failure of previous work, followed the idea that all streptococci were the same, and that the differences which the various workers had endeavored to interpret as fixed characteristics were in reality the fleeting impress of chance environment. At the same time it was asserted that these inconstant organisms produced a great variety of diseases, and to explain this varied activity the theory of elective localization was formulated. This postulates transmutations to a surprising degree of frequency, and often the agency which endows a streptococcus with affinity for the stomach, or for the heart valves, or for the joints, operates in the same focus of infection, the tonsil. Proof has been offered in the production in animals of corresponding lesions in which no factor other than the activity of streptococci is considered. Inasmuch as it is insisted that the properties of elective localization are apt to be lost in the process of isolating a streptococcus, and that the first culture even though mixed must be used, this theory in its present form eludes accurate experimental demonstration.

The other course was to attack the problem from the standpoint of biochemical reactions. Gordon (18) studied the fermentation reactions of streptococci on certain test substances. This line of work was soon taken up by others, and Andrewes and Horder (19) reported extensive studies on 1,200 strains and succeeded in placing the so called pathogenic streptococci in four groups. They found that outside of the first group called *pyogenes*, which was composed largely of streptococci from purulent processes, the source of the streptococci did not accurately determine their place in the groups. In analyzing the results of the reactions, the different groups are found to contain from 14 to 42 per cent of variants. Objections to these biochemical tests on the grounds of inconstancy were advanced by Ritchie (20), Walker (21), and others. However, the discrepancies were elicited by doing the tests under diverse conditions of age, viability, and culture media, and do not discredit the method as much as they indicate the necessity for uniformity in performing the tests. The reasons for variation have been discussed by Thro (22, 23) and Broadhurst (24). Very recently Holman (25) suggested a more extensive differentiation of the entire streptococcus group, based upon their hemolytic and methemoglobin production properties and upon their fermentation of lactose, mannite, and salicin. Sixteen groups are described, but as so few carbohydrates are employed no variants of these groups are recognizable.



Andrewes and Horder found agglutination reactions "troublesome and disappointing," but attempts were soon directed to a correlation of fermentation and agglutination reactions. Floyd and Wolbach (26) concluded that fermentation reactions could be used to separate streptococci into large groups, while agglutination reactions merely emphasized the individuality of members. Kligler (27), on the other hand, found that agglutination reactions ran strikingly parallel with fermentation reactions.

Although Schottmüller (28) in 1903 emphasized the difference between streptococci on the basis of their action upon blood agar plates, very little attention is paid to this factor in the above reports, except for Holman's work. Most workers agree in establishing a strong connection between a certain number of purulent infections and the hemolytic streptococcus (*Streptococcus pyogenes*). It is not unreasonable to assume that much of the cross agglutination reported was due to the fact that one type of streptococcus, the hemolytic, was tested, because the source of many of the strains was a purulent process. Analysis of the reports of Meyer (11) and Neufeld (13) illustrate this point. Analyzing the report of Kligler, in which attention was paid to the hemolytic property of the strains, we find support for the argument that the property of producing hemolysis is important. Kligler tested a serum produced by immunizing a rabbit against a hemolytic strain and found that it agglutinated ten out of twenty-nine hemolytic strains, none out of seventeen "green" strains, and five out of twelve indifferent strains. Thus this serum displayed a marked capacity to agglutinate hemolytic strains; a marked capacity to agglutinate indifferent strains; but no capacity to agglutinate green strains. Two sera immune to green strains agglutinated two out of twenty-nine hemolytic strains and seven out of seventeen green strains. Thus their agglutinating capacity was marked for green but weak for hemolytic strains.

These brief considerations make it logical to require that the hemolytic and non-hemolytic groups should be studied separately in an attempt to classify, and for this reason in the present work the study was limited to non-hemolytic strains alone.

#### EXPERIMENTAL.

In studying the organisms on which this report is based, attention was paid to the source, growth in plain broth, effect on ascitic-dextrose-agar, solubility in ox bile, action on red cells, according to the method used by Lyall (29), and virulence. In addition, the fermentation reactions were compared with the agglutination and complement fixation reactions.

*Methods.*

1. For testing the effect of streptococci on red cells, dilutions of a 24 hour broth culture were made in a row of small tubes, using plain broth as a diluent. Each tube contained 0.5 cc. of culture dilution, the doses being graduated as follows: the first tube, 0.5 cc. of culture; the second, 0.25 cc.; the third, 0.12 cc., etc. To each tube 0.5 cc. of 5 per cent saline suspension of sheep red blood corpuscles was added. The mixtures were read after incubation in the water bath, at 37°C. for 1 hour. Streptococci either (1) hemolyze the cells, (2) produce methemoglobin in the unhemolyzed cells, or (3) have no effect upon the cells. The method is not accurately quantitative, but the results are more nearly comparable than are those obtained with the blood agar plate method. However, for comparison both methods were employed.

2. For testing virulence, white mice were inoculated with 18 hour broth cultures in amounts of 2.0 cc., 1.0 cc., and 0.1 cc. If an organism was lethal at 0.1 cc. it was titrated further.

3. Fermentation reactions were done with litmus milk and with raffinose, inulin, salicin, and mannite as test substances. Media containing these were prepared by adding 1 per cent of the test substance to Hiss serum water. In examining the effect of the streptococci on these carbohydrates, a tube of each was inoculated with about 0.2 cc. of actively growing broth culture, incubated, and observed for 10 days.

4. For the animal immunization, a rabbit was used for each of the twenty-eight streptococci. Immunization was effected by injecting the animals intravenously with saline suspensions of killed streptococci at 4 day intervals, in doses equivalent to 10 cc. of broth culture. The injections were continued until the serum of an animal gave marked complement fixation with an antigen made from the corresponding streptococcus. Most of the animals produced good complement-fixing antibodies after six to ten injections.

The sera from the same animals were used for both complement fixation and agglutination reactions, and sera of the same or nearly the same dates were used for both reactions. In the case of complement fixations, as soon as a serum showed definite fixation with its

own antigen, it was tested on the same day with the same materials, hemolytic system, etc., against the twenty-eight antigens, and those antigens which gave fixation were noted. A few days later the animal was bled again and the serum was titrated against those antigens with which fixation was previously obtained. The above precautions were taken because it was noticed, in a few instances, that an old serum tended to give fixation with an increased number of antigens. In performing the agglutination reactions, a dextrose broth culture of a streptococcus was tested against the twenty-eight sera. The reason for this arrangement is obvious, since it was desirable to perform both tests with sera obtained at the same bleeding. All of the twenty-eight rabbits could not be immunized at the same time, and while the age of the serum does not affect agglutinating capacity, complement fixation reactions must be performed with fresh serum because variations in tests showed this to be advisable. Accordingly, while all the sera were on hand when the agglutination tests were made, owing to the uncertainty of keeping rabbits alive over a long period, complement fixation tests had to be made as soon as an animal's serum showed sufficient immunity.

5. In the complement fixation reactions various constituents were used in the following quantities: 0.1 cc. of streptococcus antigen, two units of complement and anti-sheep amboceptor, and descending amounts of immune serum, as 0.1 cc., 0.05 cc., 0.025 cc., etc. The complement-antigen-serum mixtures were made up to 1.5 cc. and incubated in the water bath, at 37°C. for 1 hour. Sensitized cells prepared by mixing 0.5 cc. of amboceptor dilution and 0.5 cc. of a 5 per cent suspension of cells were added, and the tubes again placed in the water bath at 37°C. for 1 hour before reading.

The antigens were prepared as follows. Washed sediment of a 24 hour broth culture was suspended in 5 cc. of saline, and 5 cc. of absolute alcohol were added. The resulting precipitate was separated by centrifugalization and desiccated *in vacuo*. It was then ground and weighed. 10 mg. were dissolved in 5 cc. of a 2 per cent anti-formin solution in the water bath at 56°C. and the solution was neutralized with 0.1 N sulfuric acid, with litmus paper as an indicator. The free chlorine was liberated by adding one or two drops of 5 per

cent sodium thiosulfate, the end-result being tested with potassium iodide starch paper. The solution was made up to 10 cc. with carbolyzed saline, and centrifugalized. 1 cc. of the antigen then represented 1 mg. of dried ground precipitate. This method was always followed. It therefore seems reasonably certain that the antigens in these experiments represent a constant amount of their corresponding streptococci.

6. Agglutination reactions were performed by making dilutions of serum in 0.5 cc. of dextrose broth, so that the first tube contained 0.1 cc. of serum; the second, 0.05 cc.; the third, 0.025 cc., etc. To each tube 0.5 cc. of a 24 hour dextrose broth culture was added. The mixtures were placed in the water bath at 37°C. for 2 hours, and readings were made after they had stood in the ice box over night.

#### RESULTS.

Table I contains a list of the organisms, their source, and the main characteristics as outlined above. Each of the twenty-eight strains was obtained from one colony.

It will be seen that with the exception of one organism (B1), which was possibly a contamination, all may be said to have come from pathological sources. Two diseases, acute rheumatic fever and bacterial endocarditis, are chiefly represented. In the case of tonsillitis, the streptococci indicated were from the predominating colonies. All are true streptococci as can be seen from their morphology, their action on ascitic-dextrose-agar, and their insolubility in bile. Those which produce methemoglobin in the presence of red blood cells are called green. Three of the streptococci were indifferent in their action upon red cells. The group from bacterial endocarditis is striking for its lack of virulence, while the rheumatism group is consistently more virulent. Attempts to raise the virulence of a few of the streptococci by passage through white mice were unsuccessful.

Table II shows the results of the fermentation reactions. All the organisms were tested soon after isolation. Thus the original tests were not all made at the same time or with the same lot of media. The final tests were performed at the same time and with the same

TABLE I.

Streptococcus.	Source.	Appearance in broth.	Length of chains. Diplococci.	Effect on ascitico-dextrose-agar.	Solubility in bile.	Effect on red blood cells.	Lethal dose for white mice.
1 A65	Blood culture; acute arthritic rheumatism.	Turbid (diplococci).	2-6	Precipitate.	Insoluble.	Green.	0.1 cc. of 24 hr. broth culture.
2 59F	"	"	6-20	"	"	"	0.1 cc.
3 A49	"	"	6-20	"	"	"	0.1 "
4 B38	"	"	4-20	"	"	"	Not fatal at 1 cc.
5 B39	"	Semiturbid; granular sediment.	4-20	"	"	"	" " 2 "
6 A135	"	Turbid.	2-8	"	"	"	" " 2 "
7 38D	"	"	8-10	"	"	"	0.1 cc. of 24 hr. broth culture.
8 A141	Blood culture; rheumatic endocarditis.	"	6-20	"	"	"	2 cc.
9 A119	"	"	2-8	"	"	"	Not virulent at 2 cc.
10 A179	Culture from heart valve; P. M. rheumatic endocarditis.*	"	6-12	"	"	"	2 cc.
11 B26	Acute tonsillitis.	Coarse diplococcus; turbid.	10-30	"	"	"	Not tested.
12 B23	"	Turbid.	10-20	"	"	"	Not fatal at 2 cc.
13 K	"	"	10-20	"	"	"	0.1 cc.
14 A102	Sputum; lobar pneumonia.	Coarse diplococcus; turbid.	4-20	"	"	"	0.5 "

15 B1	Surface colony on plate; acute arthritic rheumatism.	Coarse diplococcus; turbid.	10-30	Precipitate.	Insoluble.	Green	1 cc.
16 A140	Knee joint; P. M. chronic arthritis.	Turbid.	4-10	"	"	"	0.1 cc.
17 A84	Blood culture; P. M. bacterial endocarditis.	Coarse diplococcus; turbid.	4-20	"	"	"	0.5 "
18 A4	Blood culture; A. M. bacterial endocarditis.	Turbid.	6-20	"	"	"	Not fatal at 2 cc.
19 B4	"	"	2-8	"	"	"	"
20 A148	"	"	2-8	"	"	"	"
21 XK	"	"	2-8	"	"	"	"
22 A30	"	"	10-50	"	"	"	"
23 R	"	"	4-10	"	"	"	"
24 B29	"	"	2-6	"	"	"	"
25 O	"	Clear medium, soft sediment.	10-30	"	"	"	"
26 A26	"	Diplococci, bacillary shapes; semiturbid; clumps.		"	"	Indifferent.	"
27 A56	"	Clear, clumps; granular sediment.	2-6	"	"	"	"
28 MB	"	Clear; granular sediment.	2-6	"	"	"	"

\* P. M. indicates postmortem; A. M., antemortem.



TABLE II.

Streptococcus.	Interval between 1st and 2nd tests.	No. of generations between 1st and 2nd tests.	Fermentation reactions.				
			Milk.	Raffinose.	Inulin.	Salicin.	Mannite.
	<i>mos</i>						
1 A4	19	10	+*	—	—	—	—
2 O	15	4	+	—	—	—	—
3 XK	8	5	+	—	—	—	—
4 59F	30	15	+	—	—	—	—
5 A49	18	4	+	—	—	—	—
6 A141	15	5	+	—	—	—	—
7 A179	14	5	+	—	—	—	—
8 B39		One test.	+	—	—	—	—
9 A30	18	10	+	+	—	—	—
10 R	13	7	+	+	—	—	—
11 A65	16	5	+	+	—	—	—
12 B29	5	2	+	+	—	—	—
13 A148	14	5	{ +	—	+	+	—
14 A102	15	4	{ +	+	—	+	—
15 B38		One test.	+	+	+	—	—
16 B23	5	3	{ +	—	+	+	+
			{ +	+	+	+	—
17 K†	15	6	{ +	+	—	+	—
			{ +	—	—	+	—
18 MB	8	4	+	—	—	+	—
19 38D			+	—	—	+	—
20 B26	5	8	+	—	—	+	—
21 A140	15	5	{ +	—	+	+	—
			{ +	—	—	+	—
22 A119	16	6	+	—	+	+	—
23 B4	8	2	+	—	+	+	—
24 B1	8	7	—	—	+	+	—
25 A26	18	9	+	—	—	+	+
26 A135	16	6	+	—	—	+	+
27 A56	19	8	+	+	+	—	+
28 A84			{ +	+	—	+	—
			{ +	—	—	+	+

\*+ indicates acid and clot; —, no acid or clot.

† The second test of No. 17 K is probably incorrect as it was recently found that the original Strain K was lost.

media. This may be an important factor in interpreting variations. The arrangement followed in the table is based on the final tests. If an organism showed a variation on second test, the test was done

three times. In five instances, the first and second tests varied. Accordingly in these five the first reading represents one test, and the second reading represents three tests.

It will be seen that all the strains can be placed in two groups of the Andrewes and Horder classification; namely, the *fecalis* group, the five mannite fermenters, and the *salivarius* group which embraces the remaining twenty-three. The results agree with those of the above mentioned investigators in showing that the source is not a determining factor in the grouping. These streptococci could be subdivided into more groups than merely those of *salivarius* and *fecalis*, if the classification advised by Holman could be applied. But this requires testing the fermentation capacities of the various strains with lactose. This was not done. No general conclusions can be drawn from this table. It merely shows that most of the organisms belong to a group called *salivarius*, the feature of which is the fermentation of raffinose but which is permitted to contain variants that do not ferment this substance. With a series of only twenty-eight members a grouping on the basis of fermentation is not conclusive since we have to place members of widely varying fermentative activity together, in a grouping which investigation on a larger scale shows to be logical.

The results of the complement fixation reactions are striking. In the early part of this experiment, the sera tested showed a wide variation in fixing capacity. Thus, while one serum would fix only its own antigen, another would fix many more, and the results seemed accidental and confused. As the work proceeded, however, it became evident, first, that, in general, where a serum gave extensive cross fixation, its corresponding antigen was fixed by very few sera, and then only by sera of equally wide fixing capacity; and second, though to a less noticeable degree, that the sera which gave fixation with a small number of antigens corresponded to antigens which gave fixation with a large number of sera. In other words, there was an inverse ratio between the fixing capacity on the part of a serum and the capacity to be fixed on the part of the corresponding antigen. Inspection of Table III will make this clear. Serum 3 has wide fixing capacity; Antigen 3 is fixed only by sera which are nearly identical in fixing capacity with Serum 3. Again, Serum 28 gives exten-

sive fixation but Antigen 28 is fixed only by Serum 28. On the other hand, Sera 8, 11, 14, and 20 are very limited in fixing capacity; the corresponding antigens, however, are fixed by many sera.

This phenomenon occurred with sufficient regularity to constitute a fact. It is important to determine its cause. The explanation adopted was based on a consideration of the complexity of the different streptococci. It was thought that some streptococci might be more complex than others, perhaps in the structure of their chemical nucleus. If a rabbit is injected with such a streptococcus, its serum will present an antibody capable of fixing this streptococcus, and such other streptococci as contain the same or some of the same structural units. But if a rabbit is injected with an organism containing only one of these units, it will present an antibody incapable of union with the more complex structures and will fix only streptococci of corresponding simplicity. This led to the placing of some antigens above others in the table, and to the formation of subgroups.

Table III illustrates these points. Sera 3 to 7 have large fixing capacity. But Antigens 3 to 7 are fixed only by sera which have nearly equal fixing capacity: *viz.*, Nos. 3 to 7. This for the time being is called Group I. Besides fixing its own antigens this group of sera fixes Antigens 8 to 16. Of these, Nos. 8 to 12 may be said to constitute a separate group, first, because Antigens 8 to 12 are fixed by Sera 3 to 7, and second, because Sera 8 to 12 show similar though not identical relationships with antigens. Antigens 13 to 16 as well as Sera 13 to 16 show new relationships, and constitute an intermediate group, because by antigen and by antibody they are related to Groups I and II. Inspection of the table will show that these connections are irregular, but attention must be paid to these subgroups, because in a larger series of organisms such subgroups may assume large proportions. In a small series like the present one, irregular members in the intermediate positions are to be expected. Antigens 21 to 28 as well as the corresponding sera seem to constitute a group independent of Group I which is called Group II. In this group also tendency to subgroup formation can be readily seen, and no doubt, in a larger series, Nos. 22, 24, and 26 might be the heads of a number of identical organisms. In fact, No. 28 is the

only serum of sufficiently wide reacting capacity to deserve an independent grouping in the way that Sera 3 to 7 are distinct. Nos. 17 to 20 are called Group IIa because they are closely related to Group II, both by antigen and antibody, and are intermediate members because of their relations with Groups Ia and Ib. Finally Group II is seen to be connected with Group I through Nos. 1 and 2. The naming of groups is arbitrary and merely for the purpose of distinction.

The complement fixation reactions on which this grouping is based showed some variation, where the tests were repeated; but the variations that occurred were such as not to affect the grouping of the streptococcus in question. Thus Serum 11 fixed Antigens 11 and 12, while the serum of another rabbit immune to No. 11 fixed Antigens 10, 11, and 12. In like manner, two rabbits were immune to Streptococcus 3 and their sera differed only in the intensity of fixation with Antigens 11, 13, and 21. Such variations may be incidental to uncontrollable factors in the complement fixation reaction.

The sera of twelve of the twenty-eight rabbits, including Nos. 2 and 21, were found to give negative reactions before immunization was begun and hence the non-specific fixation sometimes ascribed to normal rabbit serum cannot be a factor in these tests.

It will be noted that while an antigen is usually fixed more completely by its homologous serum, the rule does not always apply, as for example, Strain 22. The same also applies to the action of sera. Some rabbits yielded sera of weak fixing power, and one, No. 18, yielded serum which gave agglutination but not complement fixation reactions.

In the table agglutination reactions outside the zone of complement fixation are indicated by an A.

Table IV shows the results of the agglutination reactions, which were less satisfactory. A strongly positive reaction was not always present between serum and the corresponding streptococcus, and cross reactions were fewer. Agglutination occurred in some cases where there had been no complement fixation reaction, but, as Table III indicates, this apparent discrepancy does not interfere with the grouping effected by complement fixation reactions.

TABLE III.  
*Results of Complement Fixation Reactions.*

Rabbit serum.	Streptococcus antigens.																			
	1 A4	2 A26	3 K	4 A56	5 MB	6 A84	7 A140	8 38D	9 A102	10 A119	11 B4	12 A148	13 B1	14 B23	15 A141	16 A135	17 NK	18 A30	19 A179	20 R
1 A4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2 A26	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
3 K	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
4 A56	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
5 MB	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
6 A84	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
7 A140	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
8 38D	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
9 A102	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
10 A119	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
11 B4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
12 A148	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
13 B1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
21 A65	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
22 59F	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
23 A49	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
24 O	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
25 B29	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
26 B38	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
27 B39	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
28 B76	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4







TABLE IV.

*Results of Agglutination Reactions.*

Rabbit serum.	Streptococci.																											
	1 A4	2 A26	3 K	4 A56	5 MB	6 A84	7 A140 38D	8 A102	9 A119	10 A119	11 B4	12 A148	13 B1	14 B23	15 A141	16 A135 XK	17 A30	18 A179	19 R	20 A65	21 59F	22 A49	23 O	24 B29	25 B38	26 B39	27 B26	28
1 A4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 A26	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4 A56	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 MB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6 A84	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7 A140	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8 38D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9 A102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10 A119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11 B4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12 A148	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13 B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14 B23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15 A141	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16 A135	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17 XK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18 A30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19 A179	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20 R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21 A65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22 59F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23 A49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24 O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25 B29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26 B38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27 B39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28 B26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ indicates marked agglutination; ±, moderate agglutination; -, no agglutination; o, test not done.

If greater reliance could be placed in the results of the agglutination tests, contrast could be pointed out between the form of the chart of agglutination reactions, and that of complement fixation reactions. This is especially noticeable in the upper left-hand corner where sera with wide fixing capacity have very narrow agglutinating capacity. But it is better to await a similar finding in the study of other groups of bacteria, as well as more definite knowledge concerning the mechanism of the two reactions in question.

It will be noticed that there is no agreement between the grouping based on fermentation reactions and that based on agglutination reactions. For example, Strains 38D and B26 ferment the test substances in the same way; yet they occupy widely separated places in the chart of agglutinations. The same can be said of Strains A26 and A135 as well as of A65 and B29. It is probable that fermentation tests and agglutination tests are instruments which measure two widely different activities of streptococci; so that classifications based on these two tests have little common ground for comparison.

#### DISCUSSION.

The simplest observation that may be made on examining the chart of complement fixation reactions is that there is a right-sided element and a left-sided element, together with a group in the mid-zone, where these elements mingle. In any arrangement of the chart, Serum 3 will always occupy a position on the side opposite the side on which Serum 28 will be. While it is true that study of more streptococci might reveal strains different from those herein tabulated, it is not probable that the general form of the chart would be changed, because there are at present no elements suggestive of a new kind of variation which further study would accentuate. A repetition of this entire experiment with the same streptococci would probably not alter the form of the chart in any way because similar reactions were found in those cases where the immunization was repeated with fresh animals and the tests were done a second time.

The next observation to be made, and one which was made first in the present work, is that the strains seem to differ in complexity

with corresponding difference in their antisera. Thus a strain which produces an antiserum of wide reacting capacity, will not itself react with a serum of narrow capacity; and a strain which produces an antiserum of narrow reacting capacity will usually react with many sera of wider reacting capacity than its own. This is the inverse ratio described above.

The work of others with other kinds of bacteria, while not expressing it directly, can be interpreted in such a way as to support both these observations. This should be the subject of another discussion. It will suffice to say that classifications of gonococci and typhoid bacilli can be arranged to show both the tendency to right-sided and left-sided features as well as the inverse ratio referred to above. Previous classifications of streptococci have not embraced more than twelve strains with corresponding antisera.

The explanation of these observations must be theoretical because the mechanism of the complement fixation reaction has not been completely determined.

If, hypothetically, we regard this reaction as an adsorption phenomenon (30), depending on differences in surface charge as well as on chemical surface configuration (31), for the combination of its different factors, it may be possible to refer to the strains on the left side of the chart as being composed of protein molecules of opposite charge to those on the right side. The complex strains would be said to be made up of several kinds of molecules of a certain charge; the simple ones of only one or two molecules of the same charge. The mid-zone would contain those strains which partake of both features. If the antisera be thought of as containing proteins of a charge opposite to that of their corresponding antigens, the failure to react with antigens of different charge can be understood. Finally, the antiserum for a complex antigen would present a complex surface configuration, while that for a simple antigen would show a corresponding simplicity.

In this way the chart of complement fixation reactions might be regarded as outlining the sphere of variation of the non-hemolytic streptococcus with regard to this one reaction.

## CONCLUSIONS.

1. No connection can be demonstrated between grouping of non-hemolytic streptococci based upon fermentation reactions, and grouping based upon immunological reactions.

2. A classification of non-hemolytic streptococci can be effected by studying the complement fixation reactions between the streptococci and their antisera.

3. The arrangement of the streptococci in such a classification depends on the fact that two diverse elements are present in the group. Some strains partake entirely of one of these elements, some entirely of the other, while other strains partake of both.

4. The arrangement is further determined by the fact that among the strains composed of one element there are differences in complexity, some strains being made up of many molecules or features, others having much simpler structure. This gives rise to an inverse ratio between the fixing capacity of a serum and the capacity of the corresponding antigen to be fixed.

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